THE CHEMISTRY AND STRUCTURE OF SOME UNUSUAL NATURALLY OCCURRING PEPTIDES

by

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TO MY PARENTS

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CONTENTS

Abstract

The object of this investigation was a greater understanding of the rearrangement of aspartic acid and asparagine in peptide chains from the usual α-form to the β-form via a cyclic imide. This process has been most often observed when these residues are N-terminal to glycine.

Bovine globin β-chain was obtained in a pure state by chromatographic separation of the globin chains from bovine haemoglobin, after removal of the haem. Both this natural product substrate, and commercially purified samples of egg-white lysozyme were digested with trypsin and the resultant peptides were separated by ion-exchange chromatography using a conventional cation exchange resin. Individual peptide-containing fractions were further separated on a macroreticular type cation exchanger with a different elution gradient. An anion exchange resin was also employed. These preparative peptide separations were monitored by colorimetric analysis of alkaline hydrolyzates of column eluates. The identity and sequence of the separated peptides were ascertained by amino acid analysis, since the primary structures of both substrates were known. The selectivity of the two types of cation exchanger was compared.

Peptides containing aspartic acid-glycine linkages were isolated and their structures investigated by electrophoresis, leucine aminopeptidase digestion, mass spectrometry and Edman degradation followed by dansylation, and the extent of rearrangement was determined.

The acetate salt of asparaginyl–glycyl ethyl ester was synthesized and the α→β rearrangement was induced by treatment with base, acid and buffers of differing concentrations and pHs. The degree of

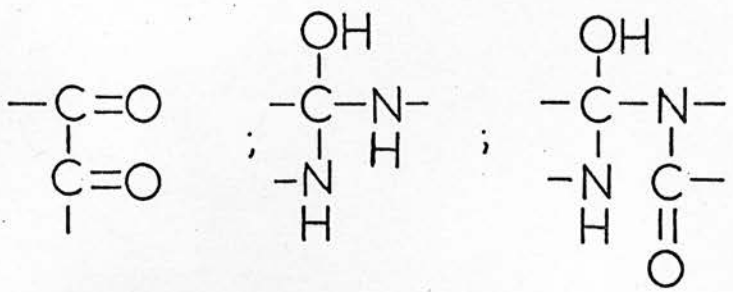
rearrangement was observed and the kinetics investigated. Treatment with leucine aminopeptidase followed by analytical ion-exchange chromatography and amino acid analysis was the method used to follow this reaction. The natural product peptides were treated similarly with base and rates of rearrangement were measured.

In the light of these results, the isolation procedure - which is responsible for the rearrangement of the natural peptides - was reappraised. An outline of a suitably efficient procedure that should not lead to excessive rearrangement of aspartic acid and asparagine is described in the text.

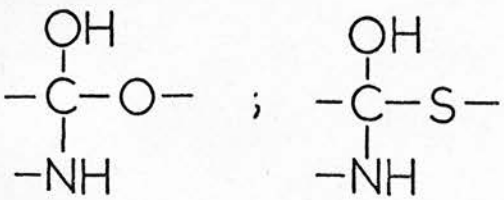
INTRODUCTION

In the currently accepted "amide theory" of peptides, the constituent amino acids are normally linked by α-peptide bonds. However, many workers have observed the presence in peptide mixtures of residues that are joined together by non-α-peptide bonds, and there has been discussion as to whether these non-α-links are present in the intact protein or whether they are caused by an α → β (or ɣ) rearrangement, induced by the conditions of the extraction and separation processes.

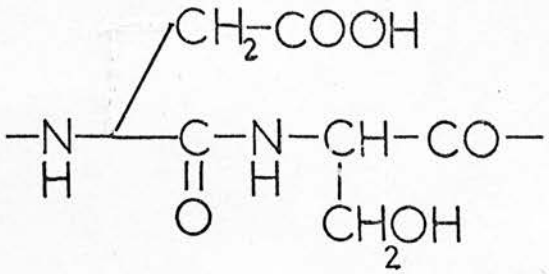
An alternative explanation has been afforded by the "Cyclol hypothesis" of peptide chain structure developed by Dorothy Wrinch[1,2,3]. This consists of two postulates. Firstly, the amino acid components are united not only in the one-bond peptide, or amide grouping, CO.NH, but also in the two-bond and three bond peptide groupings,



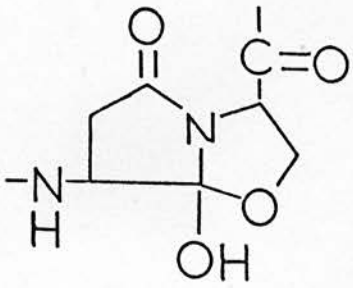
and higher peptide groupings. Secondly, reactive side groups can make ring closures; these, in the case of hydroxy and sulphydryl amino acids, introduce two-bond groupings of the form:-



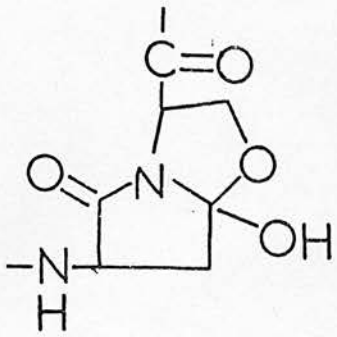
As a comparison between the two hypotheses, let us look at an aspartyl-seryl fragment. In the classical amide case it would be regarded as follows:-



In the Cyclol case we can have two forms in which both aspartyl and seryl ring closures are present.



and



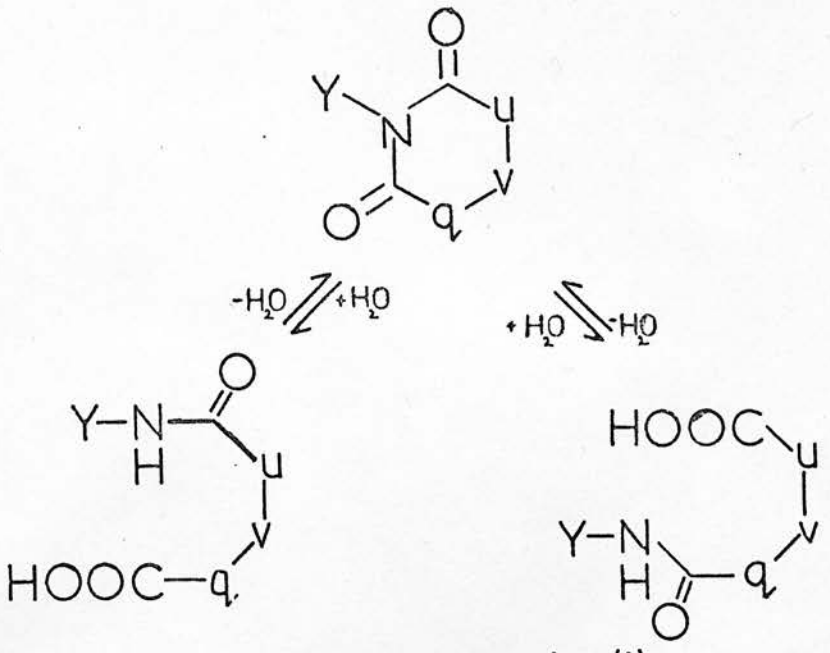
Notice that water has been eliminated in both cases.

The Cyclol hypothesis was formulated to account for various observations which are anomalous to the amide system. It lays special emphasis on the presumed lability of the multiple groupings, the reactivity of the amide grouping and the expectation that peptides are likely to undergo amine and carboxyl transfers. Each multiple bond brings a centre of instability into a peptide structure which may result in a transfer of amine or carboxyl group. This is indicated in figure A. I-VI. In I and II both bonds are endocyclic and transfers result in rearrangement. In III and IV one of the bonds is exocyclic and transfer results in synthesis or cleavage.

X = NH, O or S

Y = peptide chain N-terminal to considered residues.

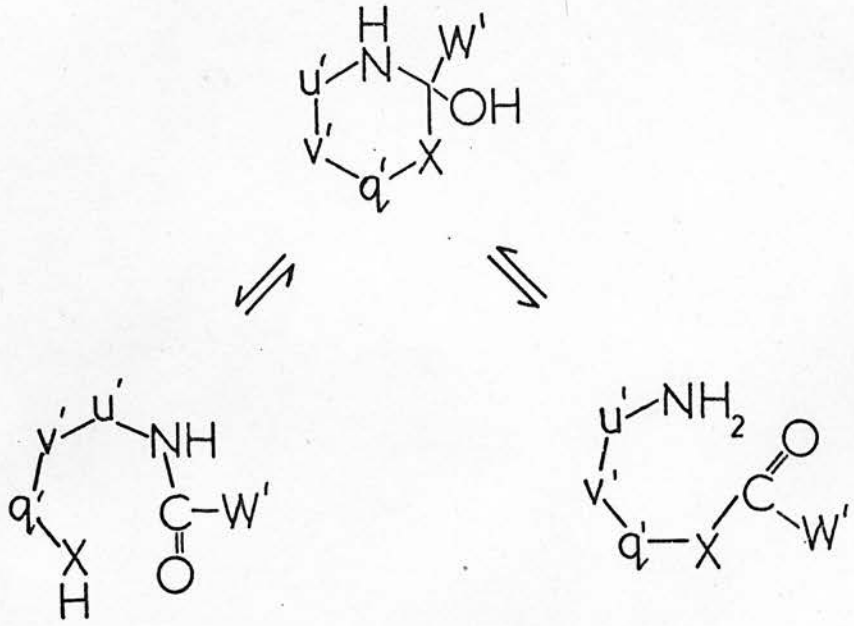
I



2-bond grouping (1)

Amine transfer

II

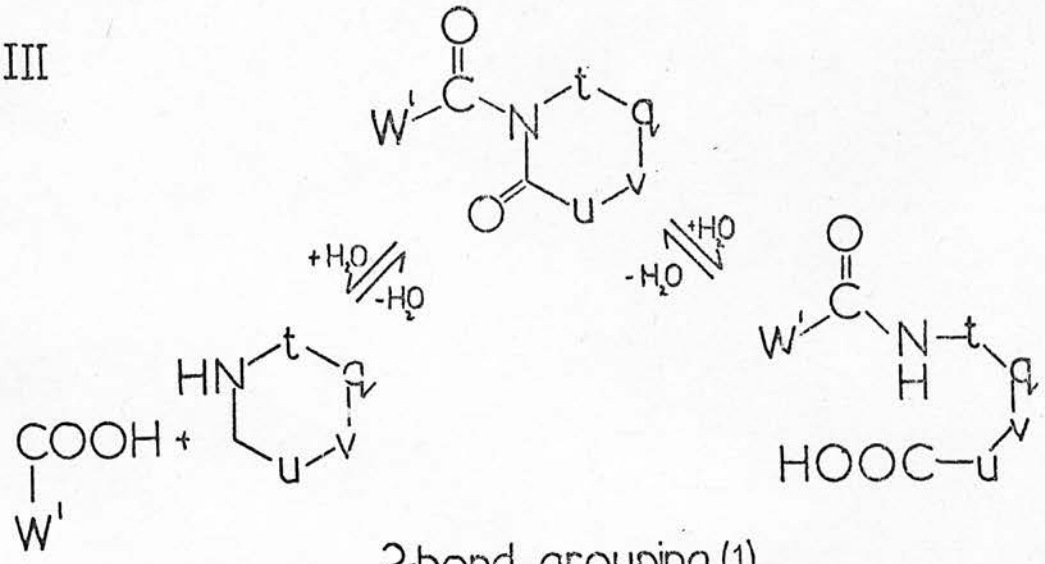


2-bond grouping (2)

Carboxyl transfer

Fig.Aa

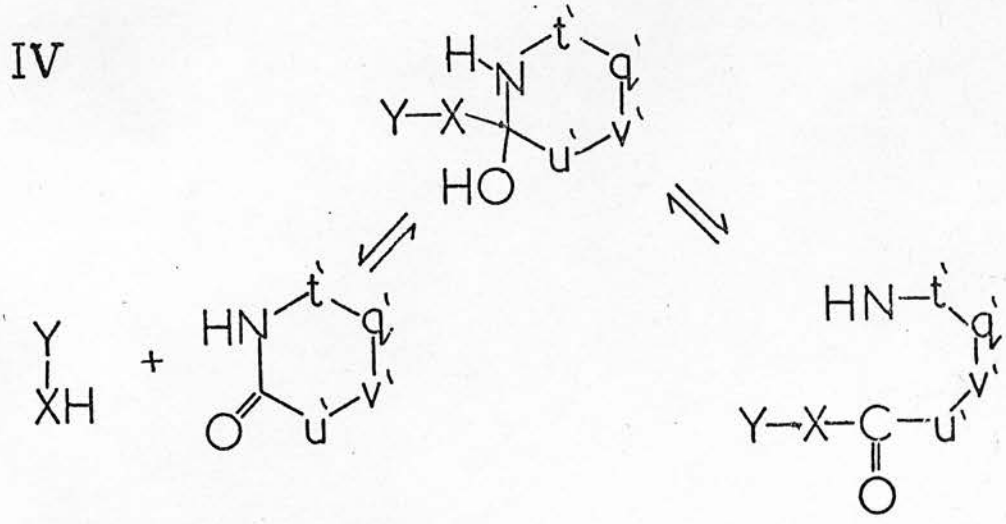
III



2-bond grouping (1)

Amine transfer

IV

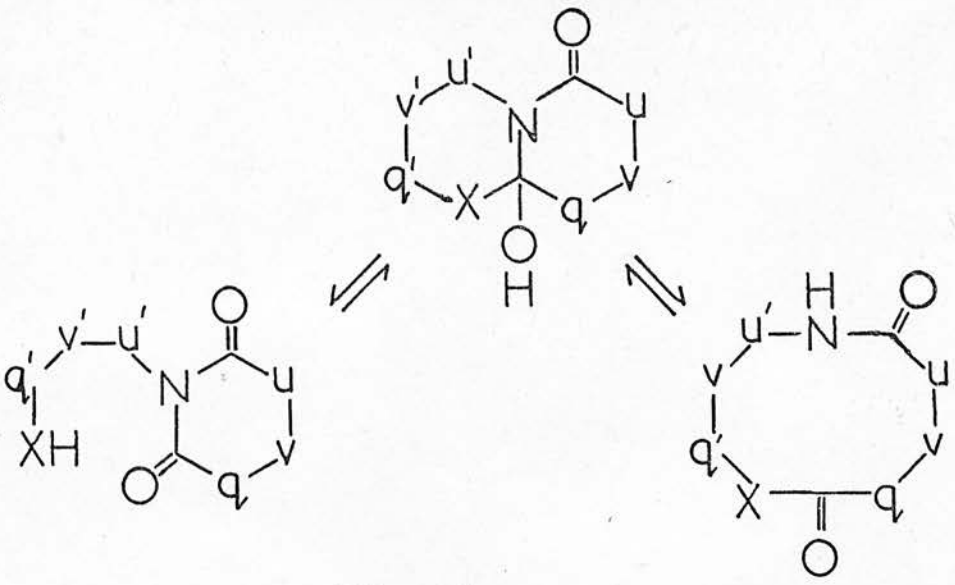


2-bond grouping (2)

Carboxyl transfer

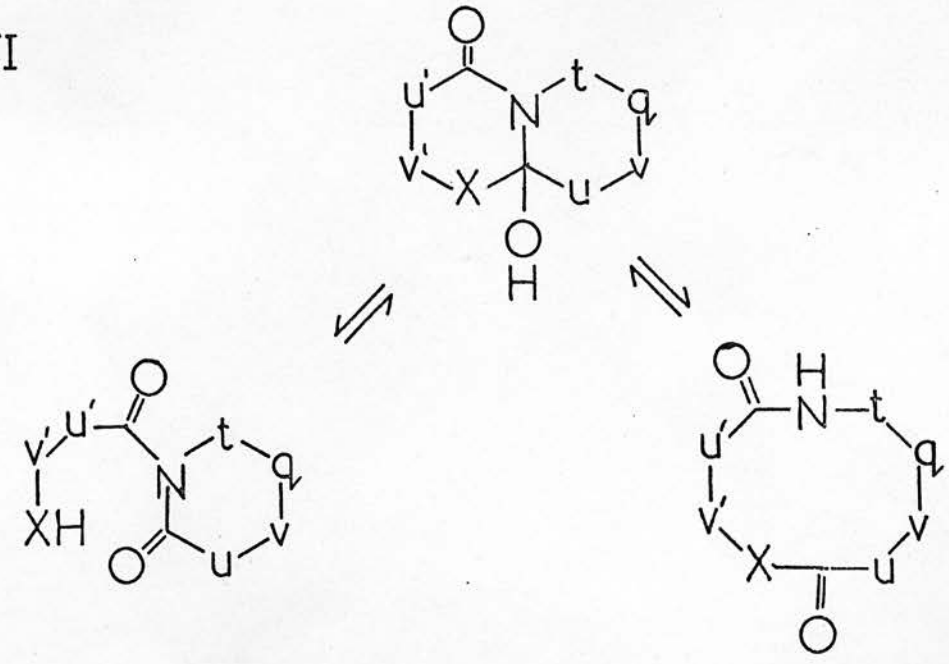
Fig.Ab

V



3-bond grouping

VI



3-bond grouping

Fig.Ac

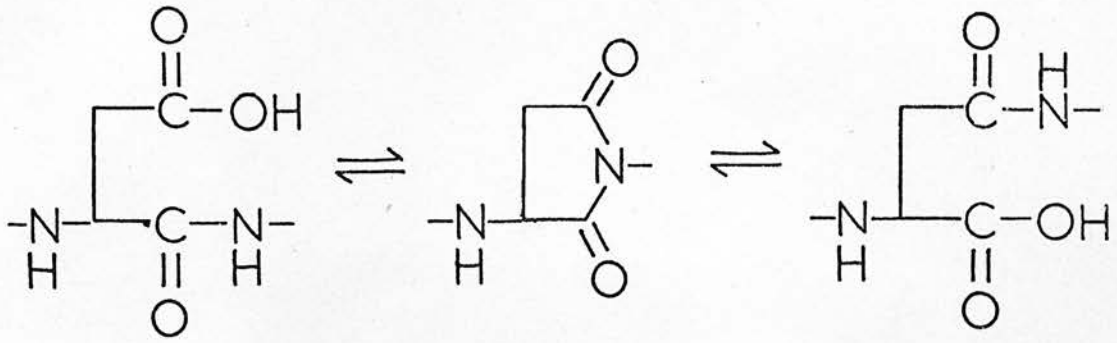
W' = peptide chain C-terminal to considered residues.

u,v,q,t,u',v',q' are to be interpreted as CH_2, CHMe, CMe_2, CEt_2 etc. The centre column ring systems need not be limited to 6 members.

The carboxyl transfer indicated in (vi) with u = v = q = t = u' = v' = CH_2 and X = O has been reported[4].

The amino acids involved in the rearrangement which is the subject of this study are asparagine and aspartic acid and glutamine and glutamic acid. The former pair can rearrange in the peptide chain to produce β-aspartyl residues and the latter pair can rearrange to γ-glutamyl residues. Loss of ammonia is observed when the two amides above undergo this reaction.

Naughton et al[5] working on trypsin, chymotrypsin and elastase observed the conversion of α-aspartyl peptides into αβ- and β-aspartyl peptides during partial acid hydrolysis (e.g. 5.7 M hydrochloric acid at 37°C for 24 hrs.). They concluded that the aspartyl residue may be in any of these three forms in the intact protein chain.



α-aspartyl αβ-aspartyl (α-aminosuccinimide) β-aspartyl

This is one of the anomalies to the amide theory mentioned previously. In the Cyclol system this is explained by postulating that the αβ-form is the natural intermediate for an α → β transition and this reversible transition is merely an expected amine transfer. The α-aminosuccinimide can be regarded as a 2-bond grouping of type (I) in fig. A.

In the above example, the aspartic acid was linked (C-N) to a serine phosphate moiety. As will be shown later, the adjacent amino acid linked to the α-carboxyl group of the rearranging amino acid (using the 'amide' system) is important in deciding whether or not the rearrangement takes place, and also, if it does, at what rate. Unless otherwise stated the classical 'amide' system will be used.

Let us consider the work of Swallow and Abraham (1958)[6] on bacitracin A and the Asp-Lys sequence in this peptide. ε-(α-L-aspartyl)-L-lysine, ε-(-β-L-aspartyl)-L-lysine, α-(α-L-aspartyl)-L-lysine, α-(β-L-aspartyl)-L-lysine and the α- and β-isohexylamides of aspartic acid were prepared, and in all cases treatment of these compounds with conc. HCl at 80°C yielded a cyclic imide derivative, αβ-aspartyl-lysine (named aminosuccinyl-lysine by Swallow and Abraham).

The -L-Asp$_{C}^{\varepsilon}\rightarrow$ Lys $\underset{N}{\overset{N}{}}$ $\overset{\alpha}{\leftarrow}$ L-Ile$_{C}$ sequence has been suggested as a part of the structure of the peptide antibiotic Bacitracin A.[7,8,9,10,11] Since the ε-aspartyl-lysine cyclization is a possible mode of formation of the natural product it will be looked at in detail. The two ε-aspartyl-lysines were treated with 11 N HCl at 80°C for between 30 minutes and 105 hours. Using paper electrophoresis at pH7 with ninhydrin detection Swallow and Abraham observed the presence of a

slow-moving base which they considered to be ε-(αβ-aspartyl)-lysine, the basic character being due to the removal of the carboxyl group of the aspartyl residue by imide formation (fig.B).

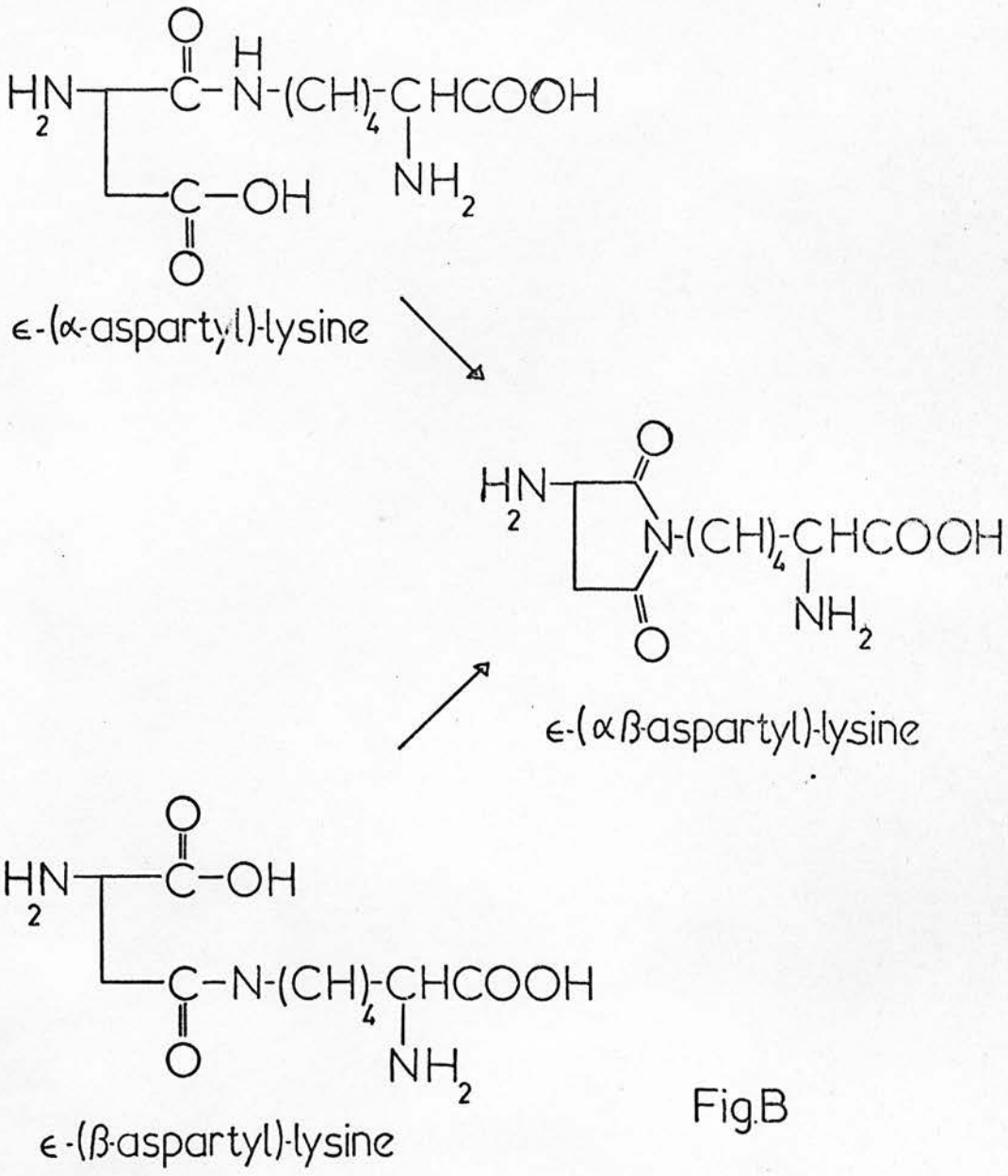


Fig.B

This view was confirmed when the substance was separated from the other products of the reaction by electrophoresis and column chromatography and some of its properties were studied. Identification was by infra-red spectrometry, amino acid analysis and formation of dinitrophenyl derivatives.

The question of possible interconversion of the α- to the β-isomer (and vice-versa) of the ε-aspartyl-lysine was checked next. Treatment of the former compound with 11 N HCl at 80°C and separation of the products of this reaction by paper electrophoresis at pH 2-3 gave spots corresponding to residual ε-(α-aspartyl)-lysine, ε-(αβ-aspartyl)-lysine, free aspartic acid and lysine and also some ε-(β-aspartyl)-lysine. The relative amounts of the α- and β- aspartyl-lysines seemed to undergo relatively little change on prolonged treatment with acid. Treating the β-isomer in the same way gave similar results except that a greater yield of the free amino acids was observed.

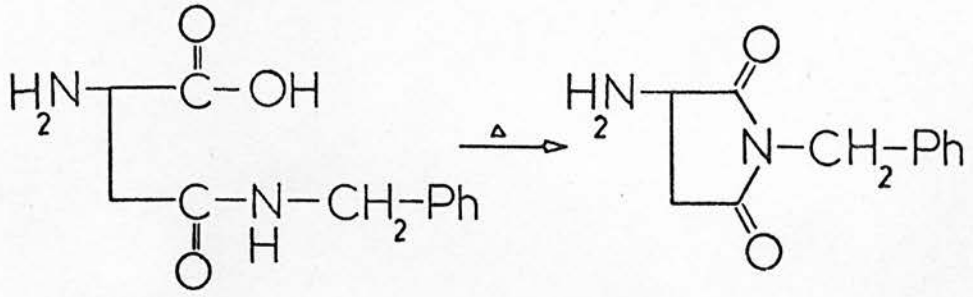
α→β isomerization again took place in 0.25 M acetic acid at 100°C but the β-isomer predominated in the reaction mixture after 24 hours. Using 0.1 N HCl at 80°C both imide formation and the rate of hydrolysis were reduced, the former more than the latter, and at 100°C the rate of accumulation of imide was small with respect to the rate of hydrolysis. The imide was observed to be relatively stable in 6 N HCl at 80°C but completely hydrolysed in 24 hours at 105°C.

Swallow and Abraham detected the compound by using infra-red spectrometry since the imide possesses a medium band at 1785cm^{-1} and a strong band at 1705cm^{-1} due to the succinimide carbonyl groups. The loss of the N-H stretch at 3300cm^{-1} from the peptide bond was also observed with the formation of the imide.

The synthetic material was only treated with acid, but the ϵ-αβ-aspartyl lysine from bacitracin A was also treated with alkali. Hydrolysis in cold, dilute alkali (0.3 M barium hydroxide at room temperature for one hour) rapidly converted the imide to a mixture of the α- and β-aspartyl-lysines of which the β-isomer greatly predominated. The ease of hydrolysis of secondary imides of this type under mild alkaline conditions is well known.[12]

The results for the α- and β-isohexylamides of aspartic acid were similar to those above, and those for the α-aspartyl-lysines differed only in that the imide, α-(αβ-aspartyl)-lysine was considerably less stable to acid than the ϵ-isomer; after 48 hours at 80°C in 11 N HCl hydrolysis to aspartic acid and lysine approached completion.

The cyclic imide intermediate is common to all these systems. Knowledge of the formation of this cyclic imide in systems of this type dates back a long way. Cherbuliez and Chambers (1924)[13] reported that benzamido-succinimide could be obtained by the action of heat on α-N-benzyl-asparagine.



Sondheimer and Holley (1954)[14,15] prepared benzyloxycarbonyl-aminosuccinimide by the saponification of N-benzyloxy-carbonyl-L-asparagine methyl ester under mild conditions. Mechanistically they assumed that the cyclization was initiated by the removal of a

proton from the amide nitrogen.

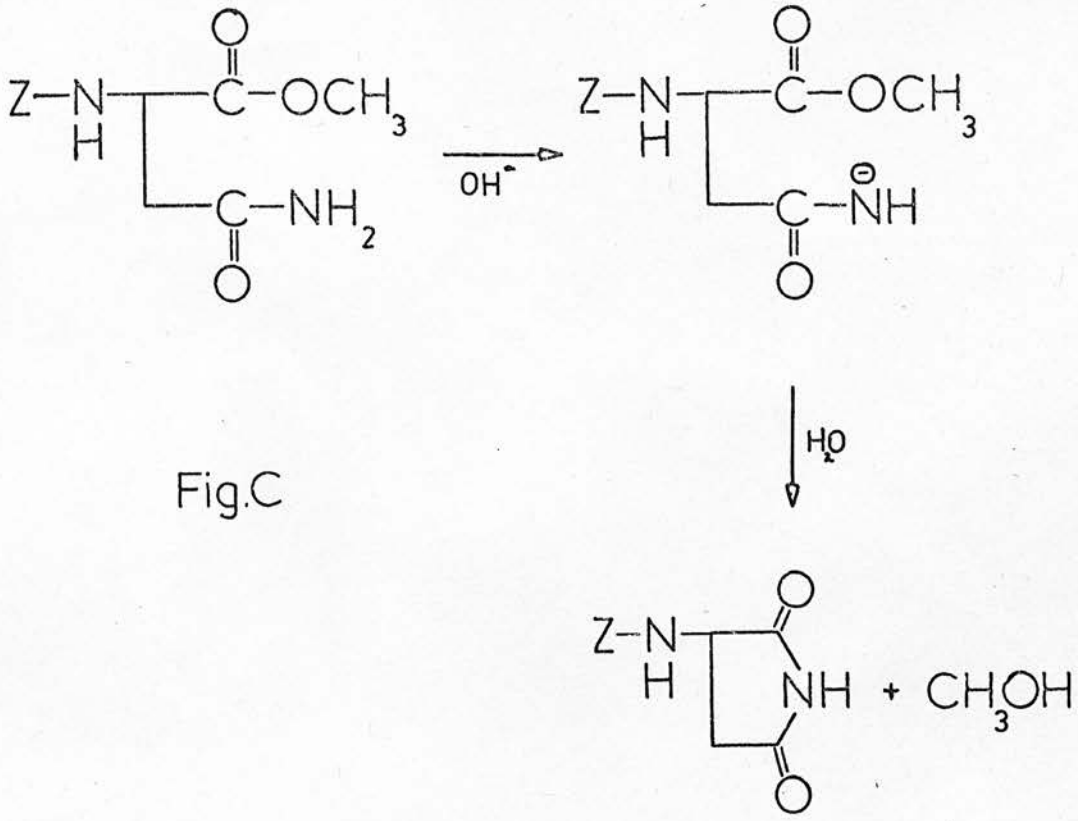
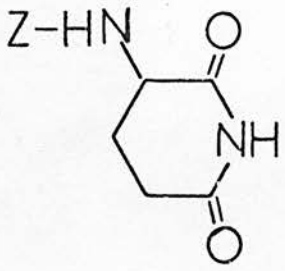


Fig.C

Z - corresponds to $C_6H_5CH_2OCO-$

Under the same conditions benzyloxy-carbonyl-glutamine methyl ester yielded the corresponding imide.



Battersby and Robinson (1955)[16] showed that the esters of N-benzoyl-α-aspartyl peptides and the corresponding β-aspartyl peptides were partly converted into αβ-aspartyl peptides on treatment with less than one equivalent of dilute aqueous sodium hydroxide.

Treatment of the imide with an excess of alkali resulted in ring opening and the formation of a mixture of N-benzoylaspartyl peptides, the β-isomer predominating. Separation was by countercurrent distribution and the structures assigned on the basis of their partition coefficient and pKa values.

They also observed that glutamyl peptides could rearrange from the α to the γ form but that certain mechanistic differences to the aspartyl system were present as might be expected since αβ-aspartyl peptides possess a 5 membered imide ring, whereas αγ-glutamyl peptides possess a 6-membered imide ring. Ring closure to the imide is the rate controlling step in the glutamyl series, whereas for the aspartyl system it is the hydrolysis of the imide.

In reactions of the imide with alkali it has been seen that the β-isomer predominates. This is to be expected since Battersby and Robinson presumed the reaction to be initiated by the nucleophilic attack of a hydroxyl group on the two carboxyl groups of the ring, and the carbon derived from the α-carboxyl group of aspartic acid will have a greater fractional positive charge, due to the electron withdrawing properties of the protonated amino group (Fig.D).

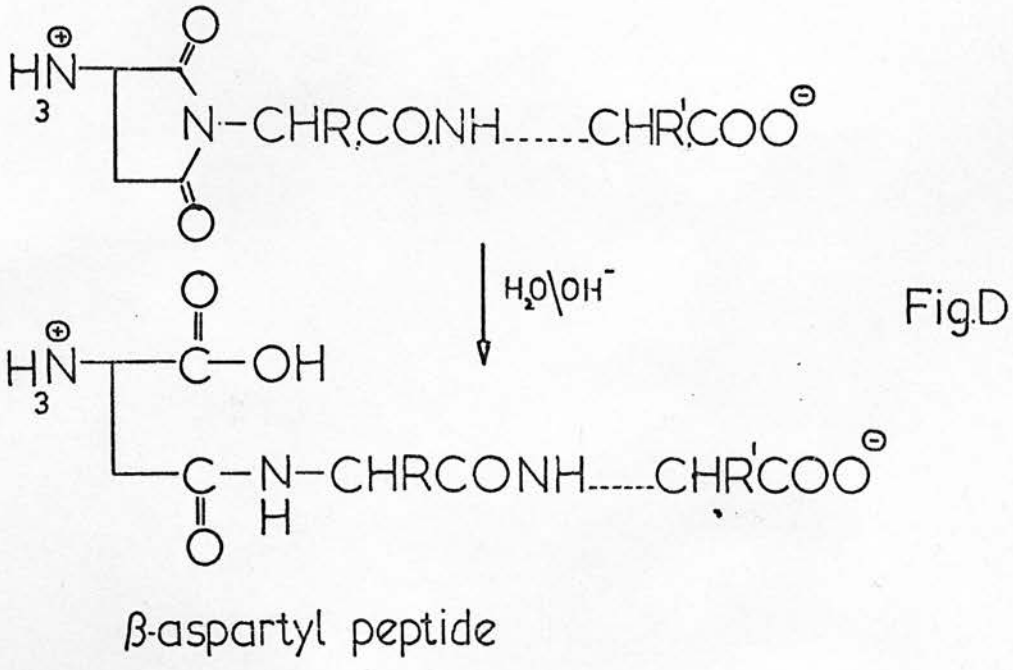


Fig.D

The work of Groskopf et al (1966)[17] agreed with this; they obtained an N-terminal β-aspartyl residue instead of an asparaginyl one after an initial enzyme digestion at pH8, but before column separation, i.e. the material had been in an alkaline environment and not come into contact with acidic buffers.

John and Young (1954)[18] reported that α-L aspartyl-valine was partly converted into the β-isomer when heated in aqueous solution at 100° for 6 hours but they did not detect any cyclic intermediate. This was probably due to the very small amount of intermediates present in the reaction mixture at any one time and the limitations of paper chromatography as a monitoring system.

Kovaks et al (1961)[19] have observed the thermal condensation of aspartic acid and noted that the product was poly-β-aspartic acid which was formed via a cyclic imide intermediate.

Let us now look at the work done on the α→β aspartyl rearrangement by Buchanan, Haley et al, who were working on urine extracts. It was noted (Haley and Buchanan, 1961)[20] that both Asp-Ser and Asp-Gly dipeptides isolated from human urine readily underwent isomerization to the β-form under acidic conditions. In 1962[21] they noted other β-aspartyl dipeptides in human urine, namely β-Asp-Thr, β-Asp-Ala, β-Asp-Ile, β-Asp-Asn, β-Asp-Val, β-Asp-Leu and β-Asp-Gln, and the tripeptides β-Asp-Gly-Val, β-Asp-Gly-Ala, β-Asp-Gly-Gly, β-Asp-Gly-Gln, β-Asp-Gly-Pro and β-Asp-Gly-Asn. Also noted were the γ-glutamyl peptides γ-Glu-Leu, γ-Glu-Ile and γ-Glu-Val. Were these peptides actually present in the urine, or were they produced during the desalting and separation procedures? These procedures involved both cation and anion exchange resins.

Buffers up to 1 M aqueous acetic acid in acidity and 1.5 M aqueous ammonium hydroxide in basicity were used in desalting, and 1M aqueous acetic acid to 1M aqueous pyridine were used in the separation procedure.

In 1966[22] Haley, et al attempted to answer the above question by searching for the source of these β-peptides in exhaustive enzymic hydrolyzates of human haemoglobin, porcine pepsin, human and bovine Achilles heal tendon collagen and egg-white lysozyme. They found that β-Asp-Gly was present in all the hydrolyzates and β-Asp-Ala was found in digestions of the haemoglobin. There was no increase in the β-Asp-Gly content of human or bovine tendon collagen with ageing in vivo nor of lysozyme in vitro. The details of this ageing experiment were as follows. The sample was divided into two, and one half was dissolved in 0.05 M Tris/HCl buffer pH 7.4 at 37℃ for 69 days. Sterile conditions were used. The other half was kept frozen at -20℃ for the same period. Both samples were brought to pH8 with sodium hydroxide and digested with hydrolytic enzymes and then analysed by paper chromatography with dipeptide controls. Both samples proved to be identical with respect to β-aspartyl peptide content. Thus, in the intact proteins tested β-aspartyl linkages, if present, do not form spontaneously from α-aspartyl or asparaginyl bonds.

The enzyme hydrolyzate from human haemoglobin indicated that 4% of the Asp-Gly linkages in the molecule were in the β-form and for Asp-Ala 0.12 - 0.24% were in the rearranged state. The lysozyme results showed that this protein had the lowest β-Asp-Gly content of any of the proteins tested. Based on 4 Asp-Gly linkages

and no Asn-Gly ones per molecule (Canfield 1963),[23] the recovery of β-peptide was only 0.6 to 0.8 μmole per gram of protein i.e. 0.2 to 0.3% of the total possible. The extent of hydrolysis was considerably less than that of the other proteins tested.

When synthetic α-Asp-Gly and Asn-Gly were incubated for 6 and 24 days under the same conditions as for the ageing experiment for lysozyme, it was found that, in the former case, 3% and 17% were converted to the β-Asp form, and in the latter case, 20% was converted to β-Asp-Gly in 4 days. No detectable conversion took place at -20°C with α-Asp-Gly.

The natural product results - since the linkages in the intact protein had been postulated as being of the α-type - and the synthetic peptide results, show that under certain conditions β-links could be formed from α-ones in both dipeptides and higher peptides. Later work[24] by Haley and Corcoran working on bovine ribonuclease showed conclusively that in this protein the β-aspartyl bonds were formed during the digestion and isolation procedures and that the bonds did not pre-exist in the native protein.

Haley and Corcoran subjected both oxidized and unoxidized forms of ribonuclease to sequential enzymic digestion (Viokase, pronase, leucine amino peptidase, prolidase and carboxypeptidases A and B) followed by ion-exchange and T.L.C. separation of the peptides. Analytical chromatography with an autoanalyser was used using the conditions of Dorer et al[25] i.e. phosphate buffer, 0.5 M in sodium at pH 1.82.

One of the peptides produced was Asn-Gly-Gln-Thr-Asn-$CySO_3H$-Tyr and it proved to be very labile.

After separation on AG50W-X2 using pyridine formate buffer pH 2.60 and 0.1 M in base, and then immediate evaporation to dryness *in vacuo*, the compound was stored overnight at -20°C. Rechromatography the next day revealed noticeable quantities of the β-compound. Incubation at 37° in 0.05 M phosphate buffer pH 7.4 for 11 days (with sterility controls) resulted in complete conversion to the β-aspartyl peptide. The lability of N-terminal asparaginyl peptides is such that the non-enzymic conversion of Asn-Gly to β-Asp-Gly occurs at ten times the rate of conversion of α-Asp-Gly to β-Asp-Gly. Ambler (1963)[26] has also noted this lability with N-terminal asparaginyl peptides. He found that the N-terminal asparagine in T9b of P-Cytochrome-551 was rearranged to give αβ-Asp under the mild conditions of electrophoresis and elution. It should be noted however that not all N-terminal asparaginyl peptides are this labile as the present study of, for instance, the acetate salt of asparaginyl-glycyl ethyl ester has shown (see later).

Returning to Haley and Corcoran's work, since the formation of a β-aspartyl link six residues removed from the site of enzyme attack (chymotrypsin) in a peptide was observed and with the ease of non-enzymic conversion of the N-terminal asparagine in the peptide mentioned above to the β-aspartyl residue, it was concluded that the rearrangement took place non-enzymatically during the digestion and separation. The finding of β-aspartyl residues with 2,3,4,7 and possibly 19 residues all matching a known sequence of bovine ribonuclease, leaves little doubt that the peptides originated from the sequence and were not the result of an adventitious attachment of a few glycine and glycyl peptide molecules to a β-carboxyl or

amide group of aspartic acid or asparagine. Rearrangement of the aspartic acid next to cysteic acid was not reported.

Thus with reference to the proteins studied by Buchanan, Haley et al, it would appear that the β-aspartyl peptides are produced from their α-analogues in the digestion and purification procedures and are not present in the native protein. A possible exception to this is in the case of bacitracin A where Swallow and Abraham (1959)[27] concluded that both the carboxyl groups of the aspartic acid directly linked to the ε-amino group of lysine, were bound. Pisano et al (1966)[28] has noted that there is the possibility of β-Asp-Gly bonds being used as cross-links for polypeptide chains. Their evidence comes from the fact that more β-Asp-Gly units were found in the highly cross-linked Achilles heel collagen than in skin collagen which contains few inter-and intra-molecular cross-links. The natural product proteins used in this study, egg-white lysozyme and bovine haemoglobin have had their structures elucidated,[23,29] and it has been shown that the Asp-Gly links are all in the α-form in the native material.

Thus far, this work has dealt mainly with Asp-Gly, Asn-Gly and Asp-Lys linkages, the former two being the most commonly encountered in the rearranged form. Mention should be made of some other β-aspartyl peptides, firstly some synthetic Asp-Ser peptide derivatives. Neurath and Hartley (1959)[30] prepared N-benzyloxycarbonyl β-benzyl-aspartyl-serine amide and wished to prepare the corresponding β-carboxylate. Benzyl esters are relatively stable to general base catalysis. They found however that even at pH7 this benzyl ester was quite readily cleaved (the $[OH^-] \sim 10^{-7}$M in 50% aqueous dioxan) with

a half-life of approximately 30 mins at pH7, 1½ mins at pH8 and 0.10mins at pH9. Relative to benzyl propionate the rate of hydrolysis of this compound was found to be 10^5-10^6 times as fast.

The primary α-amide of benzyloxycarbonyl - β-benzyl-aspartic acid under the above conditions formed a cyclic imide with the elimination of benzyl alcohol (Fig.E).

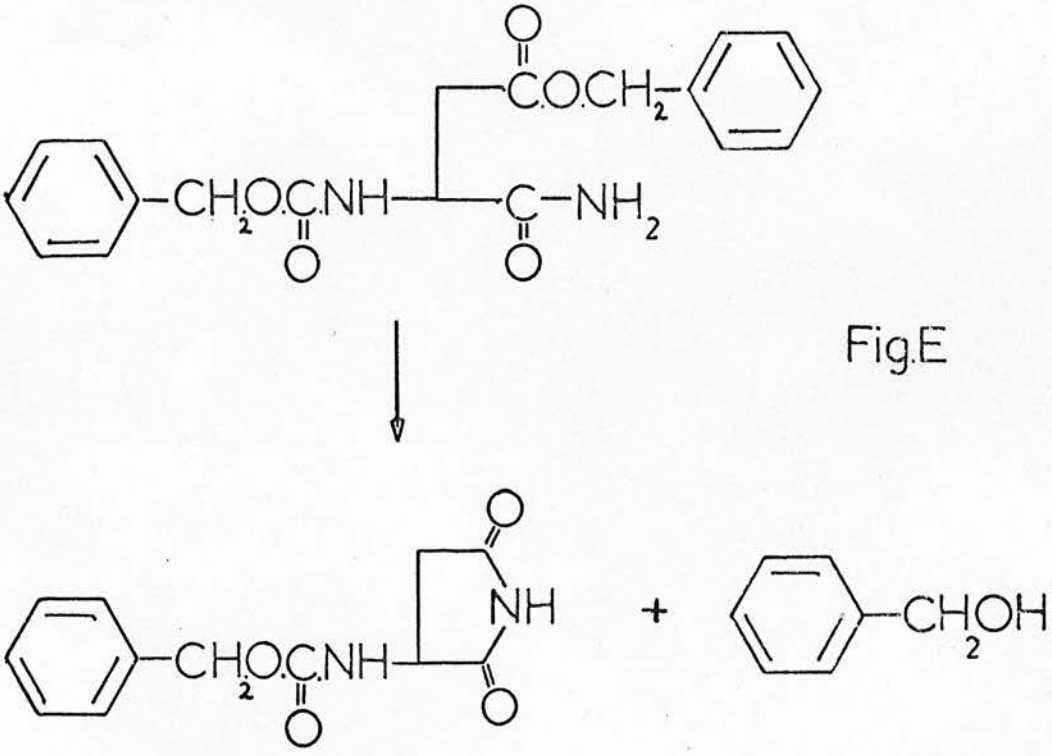


Fig.E

cf. Asp-Lys in Swallow and Abraham's work[6].

The imide was found to dissociate at pk 9.5 to give $\rangle N^-$; the presence of the negative charge makes the compound resistant to further hydroxyl-catalysed degradation to the β-carboxylate.

If instead of the primary amine, $-NH_2$, we had the secondary amine, -NHR, as in peptides, cyclization would no longer result in an ionizable imide and so secondary imides should be even more susceptible to basic hydrolysis than primary imides. The general mechanism for

hydrolysis of β-esters of aspartyl peptides is therefore:-

$$\text{ester} \xrightarrow[K_1]{} \text{imide} \xrightarrow[K_2]{} \text{carboxylate}$$

The work of Bernhard, et al (1962)[31] largely agrees with the above work. They found that β-benzyl esters of benzyloxycarbonyl-aspartyl amides and peptides underwent rapid saponification in dioxan-water mixtures. There was strong rate dependance on the nature of the particular amide or peptide, β-benzyl N-benzyloxy-carbonyl-aspartyl-serine amide being very rapid both in respect to imide formation and subsequent hydrolysis. In a 7:3 (v/v) dioxan-water mixture the second order specific rate of hydroxyl ion catalyzed formation of benzyl alcohol for this compound, exceeded by a factor of 10^7 that for benzyl propionate. This compares with a factor of 10^5-10^6 found by Neurath and Hartley. Although this rapid rate is dependent on the acidity of the peptide bond, the conformation of the molecule and the acidity of the seryl hydroxy group play important roles in the catalysis.

The imide intermediates postulated here both by Neurath and Hartley and by Bernhard were detected by optical rotation changes in the course of the reaction and by direct isolation. As stated before, Swallow and Abraham used infra-red spectrometry.

Finally let us look at the work of Piszkiewicz, Landon and Smith (1970)[32] and their results for aspartyl-proline bond cleavages. These were found to be hydrolyzed during exposure to low pH (2.5-3.5) under conditions where other aspartyl bonds are reasonably stable e.g. enzyme digestion and purification using low pH and low temperature (40°C and below) e.g. 30% acetic acid pyridine acetate buffer pH 2.80 and above. They concluded that the mechanism for the hydrolytic reaction proceeds via intramolecular catalysis by carboxylate anion

displacement of the protonated nitrogen of the peptide bond. The enhanced rate with proline was undoubtedly due to the greater basicity of the proline nitrogen (Fig.F).

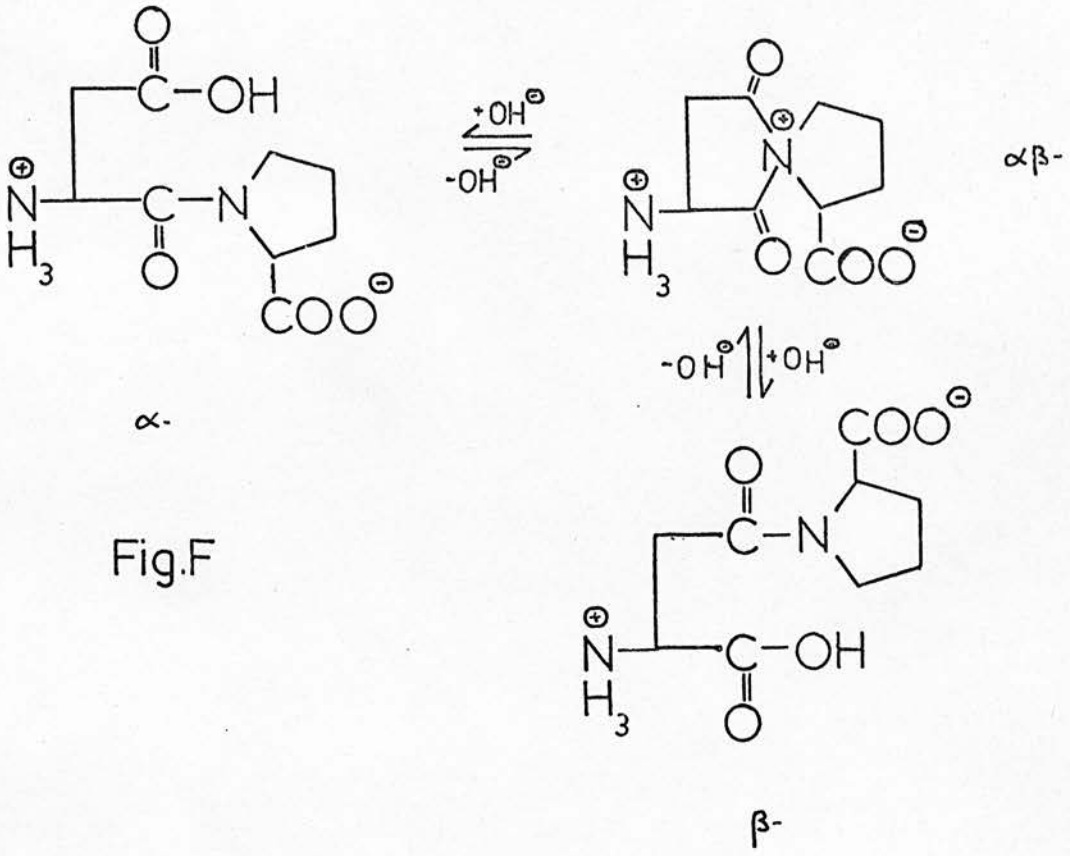


Fig.F

This work was done using bovine liver glutamate dehydrogenase after it was observed that the two Asp-Pro bonds were almost completely hydrolyzed during digestion with trypsin, pepsin and other enzymes.

It can thus be seen that aspartyl and glutamyl, and asparaginyl and glutaminyl peptides can rearrange non-enzymatically to the β-aspartyl and -glutamyl isomers. The following amino acid in the chain is important in this isomerization — α-Asp-His for instance was not found to isomerize to its β-isomer in amino acid analyses[33]. Enzymic transamination, it should be noted can also produce β-aspartyl peptides[34].

The presented work looked at the α→β rearrangement with Asp-Gly and Asn-Gly units both from natural products - egg-white lysozyme and bovine haemoglobin-and synthetic material. It was apparent that much of the previous work in the field deals qualitatively with the rearrangement, for instance estimating colour intensities with ninhydrin by eye; β-aspartyl peptides give a blue colour with ninhydrin as opposed to the standard purple colour given by their α-isomers.[18,35,36] There is little in the way of quantitative data. Haley[22] has done some kinetic measurements and concluded that Asn-Gly dipeptide under physiological conditions is converted to β-Asp-Gly at about 5% per day and α-Asp-Gly isomerized under the same conditions at the rate of about 0.5% per day.

β-Aspartyl-peptides when located can prove troublesome to analyse, for instance they are resistant to the action of many hydrolytic enzymes e.g. LAP[37]. They also resist sequential analysis by the Edman procedure - a repeating aspartic acid moiety being produced. If conditions of digestion and separation were such that rearrangement was prevented, or kept to a minimum, then sequencing would be easier and more reliable. To do this the conditions and rates of the rearrangement must be studied.

Analytical and preparative separations of the peptides and amino acids were done by chromatography on anion and cation exchange resins of both the conventional cross-linked type and also the macroreticular type. Before going on to discuss the application of ion-exchange chromatography to amino acids, peptides and proteins, a brief mention of the theory of ion-exchange and ion-exchangers may be useful.

Conventional ion-exchange molecules are polymeric skeletons insoluble in water and organic solvents which are held together by linkages crossing from one polymer chain to the next. Consider the preparation and structure of a strongly acidic cation exchange resin of the styrene-divinylbenzene type as shown in fig. G.
The greater the number of ion exchange groups per structural unit, the greater the capacity of the material. Examples of ion-exchange groups are, Rs-COOH - weakly acidic cation exchanger; Rs-SO_3H - strongly acidic cation exchanger, and for anion exchangers, $RsNH_2$- weakly basic and $Rs\overset{+}{N}R_3$ - strongly basic.

There are both synthetic resins and naturally occurring resins. In synthetic resins the whole of the particle is available for exchange whereas in some natural resins only the surfaces are available. The ions for exchange reach the centre by moving along the 'pores' and these pores are of various dimensions - generally 5-15Å but up to 40Å in conventional exchangers, and up to 1300 Å in macroreticular resins. Ions larger than this cannot exchange.
This separation of materials by size is the basis of gel-filtration.

Ion-exchange reactions are always reversible, a position of equilibrium being reached. To make the exchange go in the desired direction, the ion to be exchanged must be removed from the system - hence the use of columns.

Apart from the ion-exchange effect, resins exhibit a number of subsidiary effects which are often significant. The case of, say, a strongly acid cation exchanger will be considered; the following occurs in the course of an exchange reaction:-

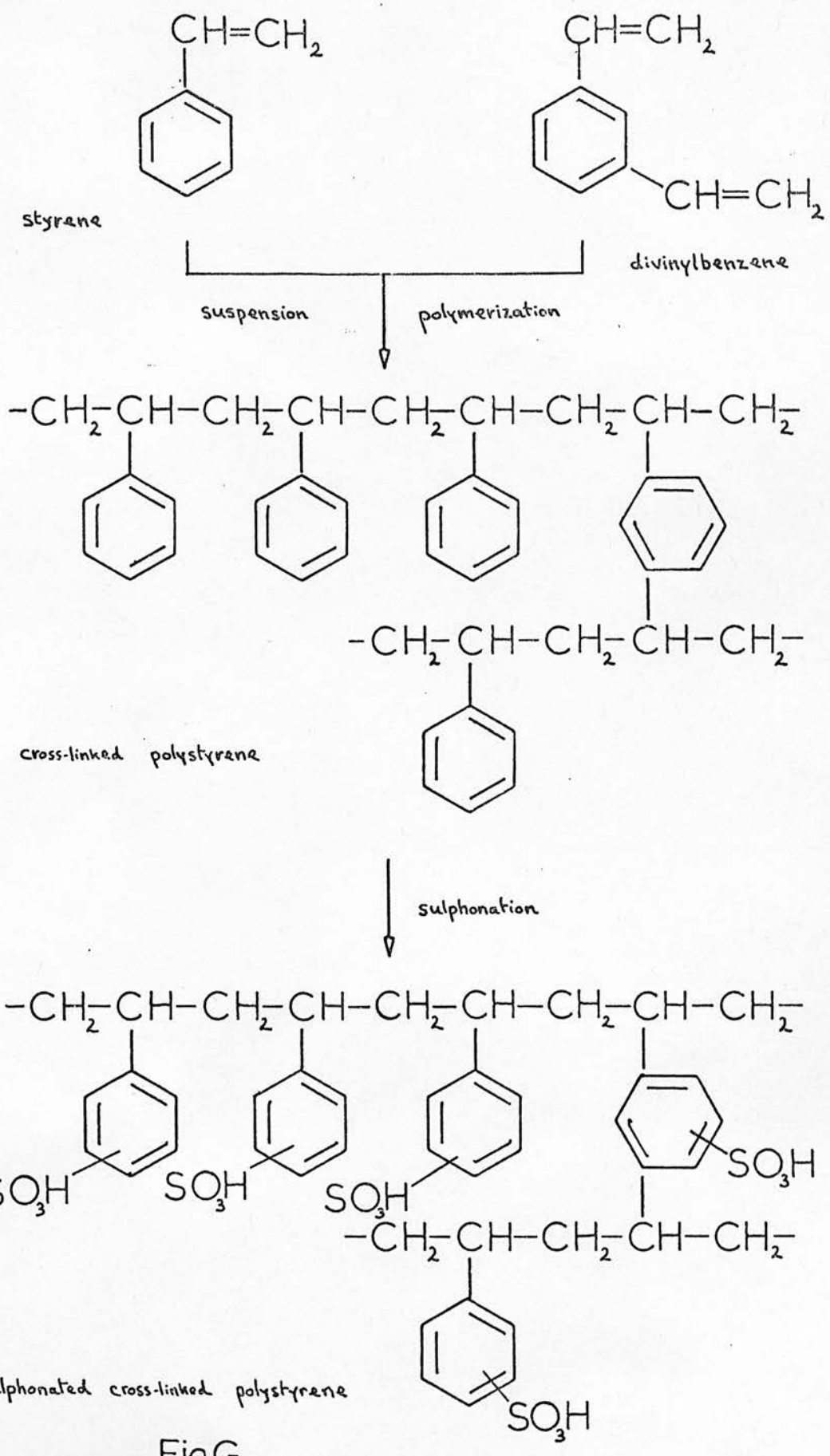

styrene
divinylbenzene
suspension
polymerization
cross-linked polystyrene
sulphonation
sulphonated cross-linked polystyrene

Fig.G

(i) Ion-exchange occurs at a measurable rate.

(ii) A selectivity of ions takes place - the resin exhibiting a preference for some ions over others.

(iii) The water content of the resin changes, and at the same time the resin swells or contracts to some extent.

(iv) Small amounts of soluble ions from the solution with the same charge as the resin are to be found inside the resin, although their concentration is much smaller than it is outside the exchanger (ion exclusion).

(v) Non-electrolytes may be taken up to some extent by the resin. These may be 'salted-out' by the addition of electrolyte.

(vi) Colloidal particles cannot penetrate the pores of the resin (ionic sieve effect) but may, if of different charge to the resin, stick on the outside.

(vii) A small heat effect (exo- or endo-) usually accompanies ion exchange.

(viii) Adsorption effects due to the hydrocarbon matrix of the resin take place.

All these phenomena take place simultaneously and are interdependent. Often however, swelling changes and the penetration of anions (in the case of a cation exchanger) into the resin can be neglected and the resin considered as an inert 'container' of exchangeable ions having a definite stoichiometric capacity, determined by the number of ionic groups in the resin structure.

Consider first selectivity. This is connected with the activity coefficient of the ion; increasing affinity for the resin corresponds to decreasing activity coefficients in solution for the

ion e.g. consider the following lyotropic series:-

(a) $Li^+ < H^+ < Na^+ < K^+ \sim NH_4^+ < Rb^+ < Cs^+ < Ag^+ < Tl^+$

Li^+ - least readily bound i.e. easiest to remove

Tl^+ - most readily bound i.e. hardest to remove

(b) $F^- < HCO_3^- < Cl^- < HSO_3^- < Br^- < NO_3^- < I^- < ClO_4^-$

The principal factors concerning these lyotropic series are firstly, hydrated ionic radius - which limits the coulombic attraction between ions - and secondly the polarizability of the ions - determining Van der Waals attraction. Selectivity increases with internal ionic concentration and this can be brought about by increasing the degree of cross-linking or increasing the number of functional groups.

Other factors influencing selectivity are:-

(i) The sieve effect i.e. exclusion of large ions. This will depend on the degree of cross-linking. There is not a sharp cut-off since the resins are elastic to some extent.

(ii) Sorption by Van der Waals forces. Where large ions are present there may be a reversal of the expected selectivity series due to Van der Waals forces of attraction. In some cases resins may sorb non-ionic molecules by Van der Waals forces.

(iii) Semi-Specific Effects. Different resins have different selectivity orders e.g. $Li^+ < Na^+ < K^+ \sim NH_4^+$ for sulphonic acid resins, but in the case of polyacrylic or polymethacrylic resins the order becomes $K^+ < Na^+ < Li^+ < NH_4^+$.

(iv) Change of selectivity with ion fraction in the resin. If the resin predominates in say, the A form, then it exhibits a

greater affinity for B than it would if it was largely in the B form. A possible explanation to this is the microheterogeneity of practical resins.

Finally, let us look briefly at the kinetics of ion-exchange. A high rate of exchange is formed by:-

(a) A resin of small particle size.

(b) Efficient mixing of the resin with the solution.

(c) High concentration of solution.

(d) A high temperature.

(e) Ions of small size.

(f) A resin of low cross-linking.

All these factors facilitate the transport of ions to, from or through the resin.

Armed with this background knowledge, the application of ion-exchange chromatography to the separation of amino acids, peptides and proteins will be considered. The separation of amino acids by means of an amino acid analyzer is good and well documented.[38,39,40] With peptides only the simpler ones can diffuse into the interior of conventional resins even of low cross-linking (limit ca 15 residues). This is not the case with macroreticular resins however. Proteins cannot get into the interior of the resin because of their large size. Separation of proteins may result from their different isoelectric points, together with different secondary adsorption forces on the surface of the resin particles. Also Van der Waals forces and hydrogen bonding probably play a part in the chromatography of proteins.

If we consider the adsorption chromatography of poly-peptides and proteins we find that it is complicated by several factors:-

(i) Their large size which prevents their entry into the conventional resin pores. Thus only the outside of the resin particle is useful and so the surface area of the resin must be increased by making the particles smaller if adequate separation is to be produced. This has the disadvantage of increasing the back-pressure in the column. The attachment of ionizing groups, e.g. carboxymethyl, to a support structure such as cellulose which has an enormous surface area not dependent on particle size, has produced adsorbants of satisfactory capacity.

(ii) Instability of the protein molecule limits the choice of solvent and eluant, temperature and pH. However even under mild solvent conditions, profound configurational changes may accompany the adsorptive and desorptive process.

(iii) Equilibrium is reached slower than with smaller molecules and the conditions for reversible equilibrium are much narrower.

(iv) For reversible adsorption of a protein to occur the number of bonds established with the adsorbant must not be too great and is dependent on the temperature, pH and ionic strength of the solution. The temperature influences the kinetics of exchange, the pH changes the number of charges on the protein and (in some cases) on the adsorbant and the ionic strength influences the dissociation of the electrostatic linkages established between protein and adsorbant; increase in the ionic strength promotes the dissociation.

A different type of resin to the conventional synthetic resin is the macroreticular or macroporous resin.[41,42] Amberlyst 15 and Amberlite 200 are examples of this type and they are produced by

suspension polymerization of styrene and divinylbenzene in the presence of a substance that is a good solvent for the monomer, but a poor solvent for the polymer (e.g. amyl alcohol). Macroreticular ion exchangers are structurally different to conventional homogeneous gels in that they have a rigid macroporous structure similar to that of conventional adsorbants, supported on the gel structure. The following points should be noted with these resins:-

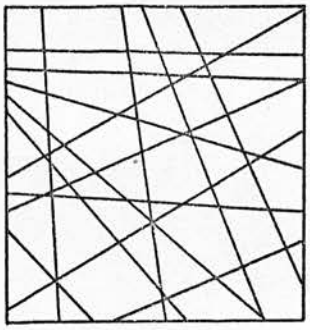
(i) The specific surface area is very large compared to a normal resin e.g. 42.5 sq.m./gm. compared to <0.1 sq.m./gm. for a conventional resin.

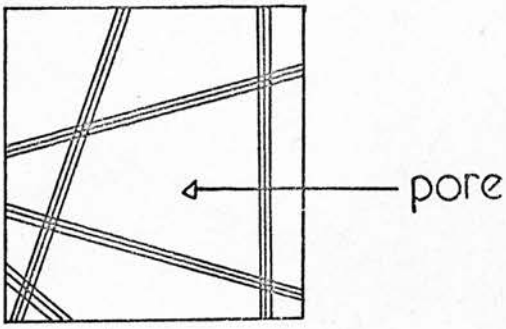
(ii) They have a definate porous structure whereas conventional resins do not have pores of this type. The distance between strands and cross-links in an ordinary resin is 5-15Å whereas the pores in macro-reticular resins can be up to 1300Å in diameter. This is the most important physical characteristic of the resin.

(iii) Macroreticular resins are much less sensitive to the nature of the solvent than the conventional resins; the difference in behaviour decreases as solvent polarity increases. Therefore there is much less likelihood of having to repack the column after every run due to compression, than is the case with standard resins.

(iv) The physical stability as measured by alternate wetting and drying, and chemical stability toward attack by oxidising agents are greater for macroreticular resins.

Returning to the porosity difference between the two types of resin, under the electron microscope the following may be seen:-





Conventional resin macroreticular resin

(The number of threads are the same in both cases)

It is difficult to speak of porosity and pore size with conventional resins since the distance between cross-links and chains of such gels vary considerably depending on such factors as electrolyte concentration, nature of the solvent, nature of the mobile and immobile ions and temperature. Since the porosity is dependent on the swelling characteristics of the gel structure, in non-aqueous, non-polar systems these resins will have a negligible pore structure. This is not the case for the macroreticular resin where some pores exist in the absence of solvent.

With their pore structure and their ability to handle larger ions, the macroreticular resins seen to be particularly suited to peptide separation. A conventional resin of low cross-linking is not a satisfactory alternative. If the degree of cross-linking is decreased in order to produce larger pores, a considerable volume change is encountered during the exchange process and during changes in ionic strength resulting in poor physical properties. The resin may have to be repacked after every run. Also, with low

cross-linked resins, the rate of chemical degradation is enhanced which results in the resin monomer (dimer etc.) being leached off the column with time and contaminating the samples.

Kunin et al[41,42] and Miller et al[43,44,45,46] in the 1960s, made clear the macroporous nature of the particles. Working on solvent modified polymer networks they were able to show that polymerization of styrene - divinylbenzene-solvent mixtures in suspension yielded copolymers whose chemical and physical properties differed radically from those of conventional resins. Depending on the proportion of solvent and of cross-linking agent, a gradation in properties from those of an expanded network to those of a macroreticular material could be obtained. The swelling properties can be predicted from the starting conditions. They found that solvent modification increases the effective porosity of the network by reducing entanglement rather than by affecting the overall covalent cross-linking.

In solvent modified resins, which include the macroreticular type, a remarkably high rate of exchange can be obtained as a result of their substantially unentangled intermolecular regions and macropores, the latter making the areas of higher cross-linking more accessible to the exchanging ions. The affect of the large pores on the ion-exchange equilibrium issmall, it being almost exclusively determined by the more highly cross-linked regions. Thus in a single resin we have the equilibrium characteristics of a highly cross-linked resin combined with the kinetic characteristics of a lower cross-linked one. The overall situation is very much more complicated however if non-solvating diluents are used instead of solvating ones

as used above.

The use of ion-exchange chromatography for separation of peptides with conventional resins is well documented[47,48,49] but there is much less literature for the case of macroreticular ones.[50] However, since Miller et al has shown that large organic molecules can exchange with these resins at rates comparable to those for small inorganic ions in conventional homogeneous gels, it would appear that their use in peptide separations will increase dramatically. When a range of solvent-modified resins from almost conventional in pore size to truly macrometicular becomes available, then it will make the job of peptide purification, simpler and more reliable.

Experimental Section

The following abbreviations will be used:-

1. Z - benzyloxycarbonyl or carbobenzoxy.
2. CM - carboxymethyl.
3. β-ae- - β-aminoethyl.
4. EDTA - ethylenediaminetetra-acetic acid.
5. DPCC - diphenylcarbonyl chloride.
6. ATEE - acetyltyrosine ethyl ester.
7. BAEE - benzylarginine ethyl ester.
8. Tris. - Tris(hydroxymethyl)-methylamine.
9. LAP - Leucine aminopeptidase.
10. T.L.C. - thin layer chromatography.
11. T.L.E. - thin layer electrophoresis.
12. OD - optical density.
13. IR - infra-red.
14. UV - ultra-violet.
15. NMR - nuclear magnetic resonnance.
16. Py. - pyridine.
17. Py. Ac. - pyridine acetate.
18. bz - benzyl.
19. NHD - ninhydrin.
20. DNS - dansyl.
21. dansyl - 1-dimethylaminonaphthalene-5-sulphonyl.
22. Ph - phenyl.
23. Amino acid abbreviations have been used in accordance with the U.I.P.A.C.-U.I.B. Commission on biochemical nomenclature recommendations (1971).

Section 1.

General Techniques

(a) Chromatography

The common feature of all chromatographic methods is the use of two phases, one stationary and one mobile; separations depend on the relative movement of these two phases. If the stationary phase is a solid, the method is known as adsorption chromatography; if it is a liquid, then as partition chromatography. The physico-chemical phenomena of adsorption and partition along with ion-exchange in the case of ion-exchange resins, are not mutually exclusive and usually more than one effect can be observed at any one time.

Paper, thin-layer and column chromatography were employed in this work with the emphasis on the latter technique.

(i) Paper Chromatography - This is a partition system where the stationary phase is water, supported by the cellulose molecules of the paper, and the mobile phase is usually a mixture of one or more organic solvents and water. In this work Whatman No.1 chromatography paper was used and a variety of solvent systems employed. A capillary was used to spot samples on the paper. The paper was dried after development with a suitable solvent and samples were detected after spraying with ninhydrin solution (0.2% ninhydrin in 90% aqueous acetone) by heating at 80°C in an air oven. Standard compounds were run as required.

(ii) Thin-layer chromatography - Glass plates were used and a thin uniform layer of known thickness of MN-Cellulose or polyamide

was applied using a Shandon Unoplan spreader. After allowing to dry at room temperature the plates were then placed in an oven at 70°C for 2 hours or so. Again a capillary was used to apply each sample and a variety of solvent systems were employed. Ninhydrin spray and ultra-violet light were used as detectors.

(iii) Column Chromatography - This can encompass partition, adsorption, ion- exchange and gel-permeation chromatography. Often more than one phenomena was taking place at the same time. Full experimental details will be given later.

(b) Electrophoresis

Electrophoresis is the separation of charged particles by differential migration in an electric field. Both paper and thin-layer electrophoresis were employed, the former using a large Miles-Hivolt 10KV unit and the latter a Desaga Heidelberg unit. Capillaries were used to apply the sample either before or after wetting the support with electrolyte depending on the concentration of the sample; in the former case a fine spray of electrolyte was directed around the spot so as to concentrate the spot rather than diffuse it. Buffers, voltages etc., will be dealt with later. Ninhydrin spray was again used as the means of detection.

(c) Spectrometry

Visible and U.V. absorption spectra were recorded on a Pye Unicam SP800 spectrophotometer and a Perkin Elmer 402. For absorption at a fixed wavelength a Pye Unicam SP500 spectrophotometer was used. One cm silica glass cells were employed and water or an appropriate buffer used in the reference cell.

Infra-red spectra were recorded on a Pye Unicam SP200 or a Perkin Elmer 247 instrument.

N.M.R. spectra were recorded on either a Varian HA60 or HA100 machine.

Mass spectrometry was performed using an AEI MS902 instrument.

(d) Measurement of pH

All pH measurements were made using either a Radiometer (Copenhagen, Denmark) type TTTlc pH meter with Scale expander or a Pye model 290 meter with scale expander. Combination electrodes were used - Radiometer, Russell (Fife, Scotland) and Ingold type 401. Readings were accurate to two decimal places whilst the third could be estimated from the expander.

(e) Freeze Drying

Freeze drying is a process whereby an aqueous solution of the sample is frozen and then subjected to a high vacuum to remove the solvent by sublimation. The technique requires less heat than ordinary evaporation and is less likely to degrade or modify peptides or proteins. The sample is left as an air-dry powder.

Samples which were not dissolved in volatile solvents were extensively dialyzed before drying to remove non-volatile salts since their presence leads to melting of frozen solutions and poor results.

The solution of protein or peptide to be dried was frozen using a Drikold/acetone bath at -78°C, in an even layer around the sides of a round-bottomed flask by rotating the flask; this provides a large surface area and reduces the drying time. The equipment used was either an Edwards Centrifugal freeze drier model 30P/599 or a small Edwards type 10P machine connected to an Edwards Speedivac ED75 oil pump.

(f) Dialysis

Dialysis removes small molecules such as salts from solutions (usually aqueous) of high molecular weight solutes such as proteins and polysaccharides by a molecular sieve action. In this laboratory, dialysis tubing (Cellulose or Visking) was boiled for about 15 minutes in distilled water to decrease the pore size and thus avoid possible loss of solute material. The tubing was tied at the bottom and about half to a third filled with the solution to be dialyzed; the space allows for water entering the tubing by osmosis. It was then tied at the top with string around a short length of glass tubing, the latter ensures that there is no build up of pressure in the bag. Dialysis was normally against running tap water, followed by several large volume changes of distilled water. About 36-48 hours were allowed for completion of dialysis.

(g) Acid Hydrolyses

Two methods of acid hydrolysis of samples for amino acid analysis were employed.

(i) The sample of peptide or protein (3 mgs) was dissolved in 5 ml of 6N constant boiling hydrochloric acid (redistilled) in a pyrex hydrolysis tube. The contents were frozen using Drikold/acetone and the tube evacuated for 10 minutes using a water pump. It was then sealed in a flame. To reduce oxidation of methionine it is advisable to allow the liquid to melt prior to sealing to free air dissolved in the hydrochloric acid. Then the tube contents were allowed to thaw out to room temperature before being

placed in a thermostatically controlled oven at 105°C for 16 hours. After this, the tube and contents were allowed to cool, then the contents frozen in Drikold/acetone and the tube opened. The frozen sample hydrolyzate was thawed and then transferred to a small flask and the hydrochloric acid removed on a rotary evaporator. The sample was redissolved in a little distilled deionized water and evaporated to dryness again. This was repeated three times to remove all the hydrochloric acid. The samples were finally dissolved in a known quantity of pH 2.20 citrate buffer containing internal standards and stored in a frozen state until required (less than a week).

(ii) The sample solution was added to a non-pyrex test tube (100 mm x 12 mm) and air dried. The tube was drawn out in a flame to produce a long, thin neck, whilst ensuring that the sample remained cool. About 1 ml of hydrochloric acid was added and excess acid trapped in the neck was removed. The sample was cooled, but not frozen, in Drikold/acetone and then the air removed from the tube by means of a water pump. After a few minutes the tube was sealed at the neck and then incubated as in (i). After allowing to cool to room temperature, the neck of the tube was broken. Acid was removed in a vacuum desiccator over sodium hydroxide pellets and phosphorus pentoxide using an oil pump with a Drikold/acetone trap. The samples were then dissolved in citrate buffer and stored as in (i).

(h) Evaporation

Three methods of evaporation of solvent were employed:-

(i) Reduced pressure evaporations were carried out using a Büchi "Rotavapor" rotary evaporator in conjunction with a water bath and water pump. A temperature of not more than 40°C was generally employed.

(ii) Air evaporation was carried out by means of the compressed air line. Two traps containing calcium chloride and a cotton wool and nylon mesh filter were employed in the line.

(iii) High vacuum evaporations were done in a vacuum desiccator over phosphorus pentoxide using an oil pump and Drikold/ acetone trap.

Peptide samples were generally concentrated by rotary evaporation, or latterly freeze-drying, then transferred from the flask to a test-tube and air dried. Finally the samples were dried in the vacuum desiccator and stored.

(i) Amino Acid Analysis

Amino acid analysis of the hydrolysed samples was carried out on a Technicon automatic amino acid analyzer. The method of analysis used was that of Spackman et al (1958)[38] as modified by Benson and Paterson (1965)[39]. For separation of the acidic and neutral amino acids a large column of Technicon "A" 25μ resin was used and separation of the basic amino acids was done using a short column of Zeo-Karb 225 12% resin. Table I.1 gives full details. Sample volumes were 0.5 ml loaded with a 500 μl Ependorf precision pipette. The internal standards used were norleucine (NOL) and L-α-amino-β-guanido-propionic acid hydrochloride (AGPA) both at a concentration of 0.1 μmole per 0.5 mls.

The peak area for each amino acid was calculated by hand measurement from the visible absorption trace on the recorder chart. Using the standard equivalent values for each amino acid - ascertained from a trace for the EEL standard - and working out the μmole values for each amino acid present, the ratio of the amino acids in the sample was calculated.

$$\mu\text{-mole value} = \frac{\text{Peak area} \times \text{NOL/AGPA equivalent}}{\text{Peak area of NOL or AGPA}} \times 0.1$$

The ninhydrin colorimetric reagent was made up using either hydrindantin or titanous chloride as antioxidising agent. Occasionally the methyl oxitol needed to be redistilled.[51,52] Acidified ferrous sulphate solution (60 gms - ferrous sulphate + 110 ml water + 6 ml concentrated sulphuric acid) was prepared and added to the methyl oxitol at a concentration of 10 mls per litre. The greenish-brown precipitate was filtered off using a water pump and the oxitol then transferred to a 5 litre round bottomed flask to which phosphoric acid (6 mls per litre) was added. The mixture was distilled, the first 100 mls were discarded and the final 100 mls of crude solution were also discarded. The redistilled oxitol was stored in dark bottles in a cool place.

(j) Peptide Analysis

An automated peptide analysis system employing alkaline hydrolysis was used.[53,54] The details were based on those of Wall (1970)[50] and the system is represented diagramatically in fig. 1.1.

N - Spot nitrogen supply.

NaOH - 2.5 M aqueous sodium hydroxide solution.

Acid - A solution of citric acid (1M) and glacial acetic acid (2M) in 30% aqueous methyl oxitol containing Brij 35 solution (5 mls/litre of stock solution comprising 1 gm per 5 mls of water).

NHD - Ninhydrin solution - kept over nitrogen in a dark bottle.

Sampler - This contained spaces for 50 samples and the sampling pattern was 45 seconds sample suction followed by 45 seconds air suction.

Proportioning Pump - Technicon, constant speed, Auto Analyzer pump.

Colorimeter - Watson-Marlow instrument set at 570 nm.

Chart Recorder - Honeywell-Brown - Y153X17V-X-GR machine or Servoscribe RE511.

Heating bath - This was a boiling water bath in which were immersed Coil I (PTFE thin wall tubing, 14 SWG, 5 min.) and Coil II (PTFE thin wall tubing, 14 SWG, 12 min.). The eluant streams from the coils were cooled in a water jacket.

The return lines were necessary since they control surging in the coils. Solvaflex and tygon tubing were used of appropriate diameter.[50]

The alkali, sample and acid mixture pH should be between pH 5.0 and 5.2.

The pattern of trace on the recorder chart was of the saw-tooth type, each peak representing a sampled fraction. It was not found to be desirable to change the pump tubing settings after 32 hours of use as was the case for the amino acid analyzer. The settings were changed after approximately 120 hours of use. The tubing was replaced when deemed necessary, the sample line more

frequently than the other lines. The peaks produced during the first few hours at a new setting were usually inferior to those after this time. A glycyl-glycine standard of known concentration was used with each run.

Both analyzers were flushed with deionized, distilled water after use and periodically cleaned with 1M hydrochloric acid followed by distilled water, then 1M sodium hydroxide solution, water and detergent solution (Decon 75). Finally the system was thoroughly rinsed with deionised, distilled water.

Table 1.1

Amino Acid Analyzer Details

	Long Column	Short Column
Resin	Technicon "A" 25 μ	Zeo-Karb 225 12%
Column Dimensions	56 x 0.636 cs	19.5 x 0.45 cms
Jacket Temp.	55℃	54℃
Sodium Concentration	0.2 N	0.48 N
Starting buffer pH	3.28 ± 0.01	5.28 ± 0.02
Second buffer pH	4.25 ± 0.02	-
Buffer Flow Rate	30 mls/hr.	30 mls/hr.
Back Pressure	100 p.s.i.	110 p.s.i.
Buffer Timer Charge	60 mins.	-
Wash buffer pH	3.28 + 10% MeOH	5.28
Regenerant	0.2N NaOH	-
Colorimeter Settings	570 nm, 570 nm, 440 nm	570 nm, 570 nm, 440 nm

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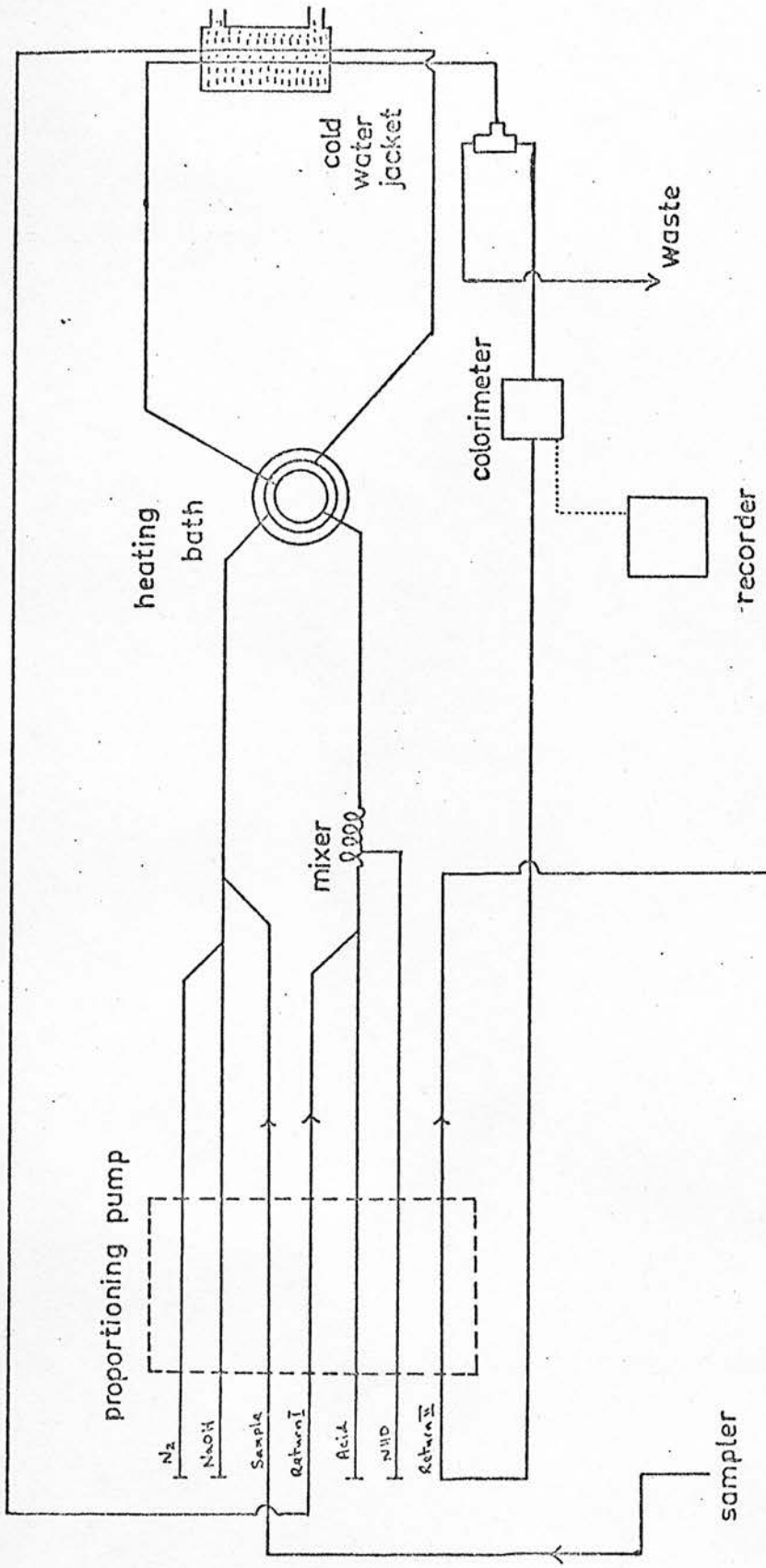


Fig.1.1

Peptide Analyzer

Section 2

Preparation of the Amberlite 200 Columns

Amberlite 200 manufactured by Rohm and Haas Company (Philadelphia) and obtained from Lennig Chemicals (Croydon) was used. It was supplied in the sodium form and technical details ascertained from the Rohm and Haas technical bulletin on Amberlite 200.

The resin cannot be used as supplied since the particles are too large; the spherical resin beads were ground into small irregular particles and these were separated hydrostatically into several fractions of narrow size range. The larger particles - up to a certain maximum size - will be used in preparative columns and the smaller size fractions for analytical columns.

(a) Grinding and Initial Fractionation

A sample of the dry resin was ground for 1½ minutes using a ball-mill and a wet paste was obtained. This was washed into a large bucket with water (4 litres). The resultant suspension was thoroughly mixed by stirring and then allowed to stand for 10 minutes. The supernatent suspension was decanted from settled large particles into another bucket and then allowed to sediment overnight. The settled particles were washed with water and collected in a sintered funnel and resuspended in water (4 litres) for 10 minutes. The supernatent suspension was decanted from settled large particles into another bucket as before and then allowed to sediment overnight. The settled particles were washed with water, filtered, air-dried and reground with more fresh resin. A grinding time of 50 seconds was found to be more suitable than 1½ minutes, since it prevented the particles from being ground too finely. The procedure was repeated until no more material sedimenting out in 10 minutes was obtained.

The resin batches collected after overnight settling were pooled and resuspended in 10 litres of water. The supernatants were discarded. After allowing to stand overnight the supernatant containing very fine particles and some fibrous impurities was removed by decantation, and the resin was collected by filtration through a sintered glass funnel (Q & Q grade A3) at the pump, washed with water and air dried. The settled volume of resin was approximately 400 mls. This was added to 4 litres of water in a 10 litre beaker and stirred for 20 minutes to break up any aggregates of resin and fully hydrate the resin. Using a combination of 2 hour and overnight settling times with 4 litre suspensions, the resin was divided into three batches. Firstly very fine material which was discarded, secondly fine material which was stored and thirdly resin for fractionation. The 'fines' were stored under dilute sodium hydroxide solution (1M) in plastic bottles. As the fractionation procedure proceeded more resin was added to this bottle. Under the microscope the resin appeared as irregularly shaped particles.

(b) Initial Purification

The resin was stirred with aqueous sodium hydroxide solution (500 mls 2M) with heating to 70°C for 20 minutes. It was then allowed to settle for 2 hours. This ensured that all clumps of resin were broken up and it was observed that the resin was much 'smoother' in appearance. When resuspended in 1½ litres of water it was not necessary to scrape the bottom of the beaker to ensure complete resuspension of solid. A magnetic stirrer was used for 2-3 minutes and then the mixture was allowed to settle for 1½ hours after which time the water appeared to be relatively clear and free from fines.

After the water was removed by decanting, aqueous nitric acid solution (500 mls, 5M) was added, the sediment was resuspended, and then warmed to 50°C for 15 minutes. The acid was then diluted by addition of distilled water to a final concentration of 1M and the material was allowed to settle overnight. After 3 hours the settling was visibly not complete. The acid was removed by decantation; the resin, now in the hydrogen form, was resuspended in cold water, stirred for 5 minutes, and left to stand overnight.

The sedimented particles of ion exchanger were finally converted back to the sodium form by addition of aqueous sodium hydroxide solution (1 litre, 2M) with stirring and heating to 70°C. After the resin had settled for 1½ hours (the sodium form of the resin settles very much faster than the hydrogen form) the aqueous supernatant was decanted and the solid was filtered and washed with water until the washings were no longer strongly alkaline. The resin was finally washed with distilled water and air dried.

(c) Fractionation

The resin was now ready to be fractionated into batches of narrow size range. The fractionation apparatus shown in fig.2.1 was used and this was assembled according to the directions of Hamilton (1958)[55].

A - Funnel where the fractionation takes place.

B - Flask into which the heavy particles fall.

C - Constant water pressure head.

D - Water flow-rate gauge.

E - Water flow rate adjusting screw

F - Water on/off tap.

G - Tap water supply - filtered with nylon mesh to remove grit algal strands etc., from the water.

H - Screw clip isolating flask B.

With screw, H, closed, the resin slurry was poured into the funnel which was half filled with water. The water supply was switched on and tap H opened. The flow rate had previously been set at 200 mls/min. A bucket was placed so as to catch the resin suspension from the side arm and the system was left until no resin remained in the funnel. All the required resin was collected in the bucket; the particles falling to the flask are too large and were mostly unground resin particles. These were stored in a plastic bottle under aqueous sodium hydroxide solution (1M) to avoid contaminating algal and fungal growth.

For subsequent, more accurate fractionations the resin was added slowly to the funnel (full of water) using the apparatus shown in fig. 2.2.

The peristaltic pump pumped air into the 2-necked bottle and resin slurry was pumped out. When the resin level was low, water was pumped into the bottle to ensure that all the resin passed into the fractionater. When all the resin had been added (which took several hours) the apparatus was left until no resin remained in the funnel. Any particles that were trapped in the funnel were allowed to fall into the flask when the water was switched off.

The water flow-rate was set at 30 mls/min for the first of the fine fractionation runs and the material collected in the flask refractionated at a higher flow rate up to 125 mls/min. Each fraction was kept separate under dilute sodium hydroxide solution until

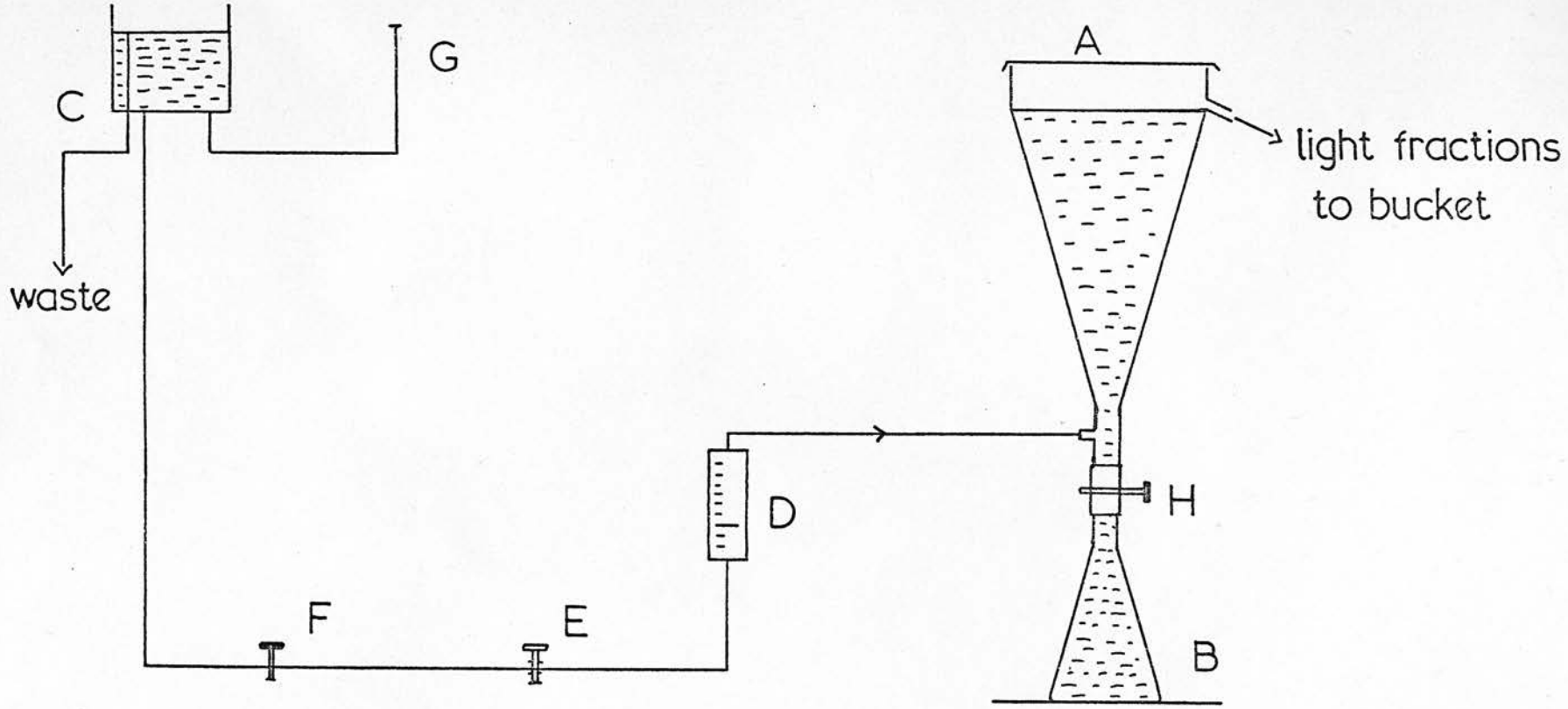


Fig.2.1

Resin Fractionater

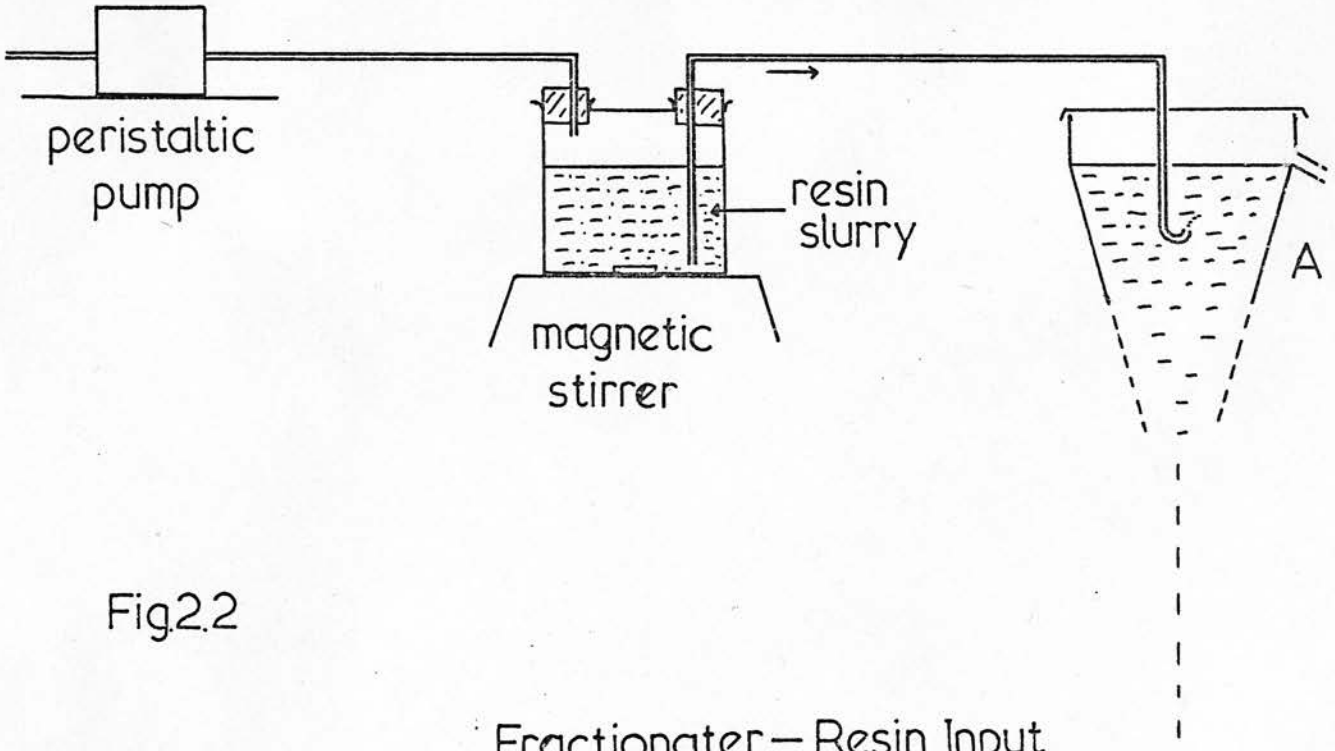


Fig.2.2

Fractionater – Resin Input

required and was observed under the microscope. Using the microscope vernier scale (12 units = 10 μ) the maximum, minimum and average maximum and minimum particle sizes were noted, and a subsequent refractionation flow rate decided upon. Fractions were combined wherever this was permissible.

(d) Fractionation Theory

Heavy particles will require a greater water flow rate than light particles to rise in the funnel. Since the density of the particles is the same, it can be said that the large particles will tend to sink and the small ones to rise. Thus by varying the flow rate various sizes of particles can be collected.

If the particles in the funnel are considered to be spheres, and act as spheres, then, by Stokes' Law:-

$$V_1 = \frac{2g\ (d_1-d_2)}{9\eta} \times 60a^2 \text{ cm/min}$$

Where

V_1 = downward linear velocity of flow

g = acceleration due to gravity

d_1 = density of the resin

d_2 = density of the water

a = radius of the particle

η = viscosity of the water

Now, for non-spheres

$V = \frac{1}{2} V$ approximately

This approximation was used to calculate theoretical maximum and minimum particle size in a given fraction.

To counteract this tendency of the small resin fragments to fall, a steady upward water flow was applied through the funnel. Since it was V-shaped, maximum flow was obtained at the narrowest part, i.e. the base, and minimum flow at the widest part, i.e. the top.

Minimum linear Velocity of flow = $\frac{\text{Input flow rate in ml per min.}}{\text{Area of a plane of water in cm}^2 \text{ at max internal diameter}}$

$$V_2 = \frac{\text{flow rate}}{r^2}$$

Units = cm/min

Particles flowed into the bucket if $V_2 > V_1$ and fell into the flask if $V_2 < V_1$. If $V_2 = V_1$ then these particles were observed in both collecting vessels.

The theory assumed that there was a uniform velocity of flow across the cross section of the funnel. This is the case under ideal conditions but in practice a slight swirl effect was always found. This was much reduced by having the water input in the neck of the funnel. A sintered disc (very porous) or a system of baffles can also help in attaining uniformity of flow.

The theoretical particle size range for two flow rates may be calculated in the following way:-

(a) 125 ml/min = 1.25×10^{-4} m^3/min.

Temperature = 15°C

r = 0.0381 m = radius of the funnel at maximum internal diameter.

g = 9.80 m/sec^2

d_1 = 1.26 kg/m^3

d_2 = 1.00 kg/m^3

η = 0.00114 kg/sec/m at 15°C

a = radius of the particle in metres

$$\frac{1.25 \times 10^{-4}}{3.81^2 \times 10^{-4}} = \frac{2 \times 9.80 \times 0.26}{9 \times 0.00114} \times 60\, a^2 \times 10^3$$

from which we see that $a^2 = 9.15 \times 10^{-10}$ m

and $a = 3.15 \times 10^{-5}$ cms = 31.5 μ

particle diameter = 63 μ

(b) flow rate = 60 mls/min

Similarly $a^2 = 4.39 \times 10^{-10}$ m and a = 20.5μ

particle diameter = 41μ

Thus the particle size range for the 60-125 ml/min fraction should be 41 μ to 63 μ.

In practice, the first fractionation for this flow rate span gave a range of between 12 μ and 82 μ. After refractionation the range was narrowed to 31-66 μ with an average size of about 50 μ.

Refractionation was repeated for flow rates between 4 and 125 ml/min until an acceptably narrow size range had been obtained, i.e. not more than a 25% spread from the mean value e.g. for a mean value of 22 μ the range was 17-27 μ.

It was observed that fibrous material occasionally appeared in the fractionation system - presumably from the tap water - and resin particles tended to adhere to these fibres. When this occurred the resin was washed with aqueous alkali (2M) (hot if necessary) and rinsed thoroughly with water before being refractionated. Also, resin was sometimes seen to stick to the sides of the funnel - albeit not very firmly - and then the entire unit (including piping) was cleaned with water and 2N mineral acid.

Eventually the following fractions were collected and stored under dilute alkali.

(a) 'fines' fraction i.e. <11 μ

(b) Size 10-17 μ - labelled 1.

(c) Size 10-26 μ with average size 13-21 μ - labelled 2.

(d) Size 17-27 μ with average size 22μ - labelled 3.

(e) Size 18-35 μ with average size 23-33 μ - labelled 4.

(f) Size 22-89 μ with average 29-50 μ

(g) Size 31-73 μ with average 45 - 68 μ

(h) Fraction requiring regrinding - large particles.

The four fractions 1-4 are suitable for use in columns; those greater than about 30 μ in diameter are too large for efficient separations and those smaller than about 10 μ cause too high a back-pressure under normal operating conditions.

(e) Final Purification

The four immediately useful fractions were finally treated in the following way to purify them ready for column packing. In turn, they were washed with distilled water and cycled three times between sodium and hydrogen forms using hot (finally boiling) aqueous sodium hydroxide solution (2M) containing 1% EDTA and cold aqueous nitric acid (5M). Distilled water wastes were incorporated in the cycling procedure. Final traces of contaminating fibrous material were removed by boiling with 4 M sodium hydroxide solution. Hot concentrated alkali dissolves cellulose and EDTA forms complexes with heavy metals present in the mixture and these were removed by washing with water. Heavy metal atoms can complicate

amino acid and peptide analysis by forming complexes with the peptide. Analytical grade reagents and a plastic spatula were used in the purification steps.

The resin, now in the sodium form, was converted to the hydrogen form with nitric acid and then washed very thoroughly with 5 M pyridine (redistilled from ninhydrin). The pyridine was allowed to drain through the resin under gravity until the washings were no longer coloured. Finally, the resin was freed from fines by allowing it to settle in 5M pyridine and decanting off the supernatent liquid. Friction of resin particles with each other or with a spatula tends to produce fines all the time and these need to be removed periodically. Batches 1 and 4 were stored and batches 2 and 3 used as described in the next section.

Section 3

Column Packing Techniques and Buffer Purification

Brief mention of the packing procedure and the buffers used will be made in this section. Amberlite 200 batch 2 was used for analytical columns and batch 3 for a preparative column. They were equilibrated in starting buffer (0.1 M pyridine acetate pH 3.1) and defined twice. Finally a slurry was made by suspending the resin in three times its own volume of buffer. Each column was packed in a standard stepwise manner such that layers of resin were formed in the column. With ground resins it is advantageous to have these layers quite small so that the resin is optimally packed and settling does not occur with time. The flow rate was quite high initially (ca. 125 mls/h) and was reduced as the column lengthened and the back-pressure increased. A final, measured flow rate of 25 mls/h was used. The columns were pumped for an hour or so with starting buffer before being used.

Zeo-Karb 225 8% cross-linked cation exchange resin of average particle size 40 μ was also packed into a preparative column. The purification and packing procedure were the same as for the Amberlite 200 resin.

An anion exchange column of "Deacidite" FFIP (<200 mesh, 2-3% cross-linked) was also prepared. The resin was washed with water and cycled several times between the chloride and hydroxide forms using cold aqueous sodium hydroxide (2M) and cold aqueous hydrochloric acid (2M). It was then heated with hydrochloric acid (2M at 70°C), washed with water and the converted to the hydroxide form. Excess alkali was removed by washing and then the resin was left to stand in 50% aqueous acetic acid overnight to convert it to the acetate form. After washing with water, the resin was suspended

in 5 litres of water and left to stand for 10 minutes. The large particles that had settled out were removed and the resin was filtered, washed, air dried and then suspended in 200 mls of pH 9.4 starting buffer. After removal of fines the resin was suspended in three times its own volume of pH 9.4 starting buffer and the suspension de-gassed. The column was picked at 37°C under slight negative pressure. This was done to remove all air from the system. The anion exchanger was much more difficult to pack satisfactorily than the cation exchangers.

From time to time the heavily-used cation exchange columns needed to be repacked since, apart from miscellaneous 'rubbish' bound to the column, polymeric material from the column was leached off it with organic buffers. The above purification procedure was used after the resin had been steeped in 5 M pyridine for a couple of days. The resins were again defined before packing. The column tops were removed - for later purification - and replaced by fresh resin when it was considered necessary.

Use of volatile buffers simplifies preparative ion-exchange chromatography since sample fractions of column eluate need not be desalted by other techniques than evaporation. In this laboratory pyridine and triethylamine formate and acetate were used for the cation exchange resins and pyridine, α-picoline and N-methyl morpholine acetate and acetic acid used for the anion exchange resin. The bases were redistilled from ninhydrin (1 gm per litre) and the acids were of analytical grade. However the triethylamine base proved unsatisfactory, the buffer persistantly giving a blue spot with ninhydrin on chromatography paper.

The base was refluxed with ninhydrin for 1 hour and then refluxed with p-toluene sulphonic acid plus p-toluene sulphonyl chloride (both 1 gm per litre) for a further hour before distilling. The acid should reduce any amide present to the amine and this would react with either the chloride or the ninhydrin. The base alone gave no blue colour on its own with ninhydrin, nor did a sample of redistilled analytical grade Analar formic acid, but on mixing the two, the blue spot was again observed with ninhydrin. Another difficulty with the buffer was that it gave a highly viscous material on concentration with a rotary evaporator and the material was very difficult to freeze dry. Since a high vacuum evaporator was not available, it was decided to use only pyridine buffers with the cation exchangers.

Section 4

Structure of Egg-White Lysozyme and Bovine Haemoglobin

The primary structure of egg-white lysozyme was determined by Canfield (1963)[23]. It was found that the single polypeptide chain, 129 amino acid residues long, has the following composition:-

Asp - 8	Gly - 12	Phe - 3
Asn - 13	Ala - 12	Lys - 6
Thr - 8	Val - 6	His - 1
Ser - 9	Met - 2	Arg - 11
Glu - 2	Ile - 6	Trp - 6
Gln - 3	Leu - 8	Cys - 4
Pro - 2	Tyr - 3	

It contains 4 Asp-Gly links per chain but no Asn-Gly links. The tryptic peptides possessing this sequence, and hence those of most interest to this study, are:-

T10 - Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Glu-Ile-Asn-Ser-Arg

T12 - Asn-Asp-Gly-Arg

T19 - Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg

Much work has been done on human and animal haemoglobins[30] by such people as Schroeder, Jones, Braunitzer and Perutz and many sequencies have been fully or partially ascertained. Generally, haemoglobins contain two types of chain, the α and the β and two α and β units are arranged in a tetrahedral structure in the molecule. Each is bound to a porphyrin ring containing an iron atom probably via two histidyl residues[56]. In the case of adult bovine haemoglobin the two α-chains are identical but the two β-chains differ in 3 residues. The total number of residues in each chain is 145 and the amino acid composition is as follows:-

α-chain -

Asp	8	Gly	9	Phe	7
Asn	3	Ala	20	Lys	11
Thr	8	Val	12	His	10
Ser	13	Met	1	Arg	3
Glu	5	Ile	20	Trp	1
Gln	1	Leu	0	Cys	0
Pro	6	Tyr	3		

β-chain -

	β^A	β^B		β^A	β^B		β^A	β^B
Asp	12	12	Gly	11	10	Phe	10	10
Asn	4	5	Ala	16	16	Lyr	13	11
Thr	6	6	Val	18	18	His	6	7
Ser	5	6	Met	3	3	Arg	4	4
Glu	9	9	Ile	0	0	Trp	2	2
Gln	2	2	Leu	17	17	Cys	1	1
Pro	4	4	Tyr	2	2			

The tryptic peptide of most interest is βT9 and it has the structure:-

Val-Leu-Asp-Ser-Phe-Ser(Asp,Gly,Met,Lys)[57]

Since we are dealing with a tryptic peptide the lysine must be C-terminal. If we compare this part of the bovine β-chain structure with that of human Hb_A β-chain and horse β-chain, the following similarities are observed.

Bovine: Val-Leu-Asp-Ser-Phe-Ser_1(Asp^7,Gly^8,Met^9,Lys)-His-

Human Hb_A: Val-Leu-Gly-Ala-Phe-Ser-Asp^7-Gly^8-Leu^9-Ala-His-

Horse: Val-Leu-His-Ser-Phe-Gly-Glu^7-Gly^8-Val^9-His-His-

The human Hb_A chain and horse chain have been fully elucidated. The sequence Ser-Asp-Gly exists in human Hb_A β-chain and in both the human case and the horse case, residue 8 in the peptide is glycine. Since globin chains are basically similar with small differences, rather than different but with small similarities, the most likely structure for the bovine βT9 globin peptide is Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met-Lys. It should be noted that the β^A and the β^B chains are identical in this region.

The entire sequence of aminoethyl-lysozyme and aminoethyl-bovine β-chain globin is given in the discussion section (figs 8.17-19) and also in Section 7 in the form of their tryptic peptides.

Section 5

Disc Electrophoresis

This technique was used to check the purity of the lysozyme and the bovine globin (see section 6). A polyacrylamide gel column was produced in glass tubes (7.5 cms x 0.4 cms) and was composed of two layers; a small pore gel in which electrophoretic separation took place and on top a small amount of a large pore gel containing the sample. The large pore gel served to prevent convection taking place. Electrophoresis was performed in a vertical position, the large pore gel containing the sample was in contact with the upper buffer reservoir, and the lower end of the small pore gel was immersed in the buffer solution in the lower reservoir. Electrodes were placed in each reservoir and the polarity set so that sample ions moved downwards through the small-pore gel. After applying the voltage for a suitable time the gels were removed from the containers and placed in a solution of fixative and protein stain. Excess dye was removed from the gels by washing in 7% aqueous acetic acid solution and by electrophoretic destaining.

A Shandon (England) unit was employed using the method given in the manufacturers instruction manual. Details are given in table 5.1.

The results (shown in table 5.2) showed that lysozyme produced one band and the bovine globin two bands under the operating conditions. Two runs for each were performed. Two blanks were run and here only one very faint band at the bottom of the gel of bromophenol blue marker was observed. The two bands for globin are presumably due to the α- and β-chains. The bands were rather diffuse.

Table 5.1

Disc Electrophoresis Details

(i) Gel Composition

(a) Small Pore Gel

1 part { Aqueous hydrochloric acid (1M) - 48 mls
Tris - 36.6 gms
N,N,N,N -tetramethylethylamine-diamine - 0.46 mls
Water - to 100 mls

1 part { Acrylamide - 10.0 gms
2 parts { N,N -methylenebisacrylamide - 2.5 gms
Water - to 100 mls

1 part { Water

1 part { Freshly made aqueous solution of ammonium persulphate (1.4 gms/litre)

(b) Large pore bead form polyacrylamide Gel

Biogel-P300, preswollen with 20% aqueous sucrose.

(ii) Electrophoretic Buffer Solutions (both reservoirs)

$^{1}/_{10}$" aqueous solution of Tris-Glycine buffer (Tris buffer 6.0 gms, Glycine 28.8 gms and Water to one litre).

(iii) Gel marker Dye - bromophenol blue.

(iv) Protein Stain - naphthol blue-black (1 gm in 100 mls of 7% aqueous acetic acid).

(v) Electrophoretic current - 5ma per tube.

(vi) Electrostatic destaining - current 5-15 ma per tube in 7% aqueous acetic acid.

(viii) Time - determined by progress of the marker dye (ca 30 minutes).

Table 5.2

Disc Electrophoresis Results

Protein	No. of Protein Bands.	Distance travelled by Marker Dye, m	Distance travelled by Protein, p	p/m x 100%
Lysozyme(i)	1	4.5 cms	3.5-3.7 cms	78-82%
" (ii)	1	4.4 cms	3.4-3.6 cms	77-82%
Bovine Globin(i)	2	4.75 cms	3.0 & 3.2 cms	63 & 68%
Bovine Globin(ii)	2	3.9 cms	2.4 & 2.6 cms	62 & 67%
Blank (i)	0	3.5 cms	-	-
" (ii)	0	3.6 cms	-	-

Section 6

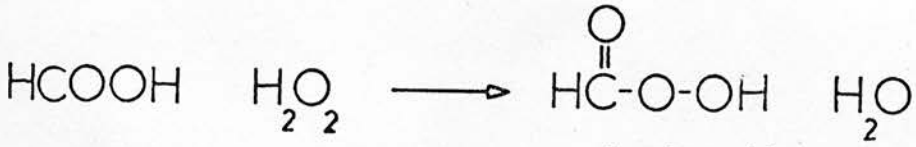
Chemical Modification of Natural Product Substrates

(a) Preparation of Modified Lysozyme

The lysozyme was egg-white lysozyme chloride, twice recrystallized, obtained from Sigma. The 129 residue, polypeptide chain is not free to unfold, but is anchored into a more or less specific shape by the four disulphide bridges joining the cysteine residues and also by hydrogen bonding. To ensure complete enzymic digestion of the lysozyme it was necessary to cleave and block the disulphide bonds and break the hydrogen bonds. The latter was easily accomplished by dissolving the protein in 9M urea solution. Three methods of disulphide bond cleavage were tried[58] and the most satisfactory method was the mercaptoethanol-ethyleneimine technique.

(i) Performic Acid Oxidation[59]

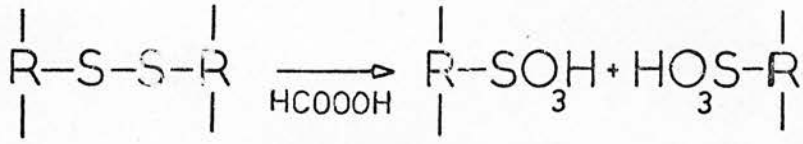
The oxidative method of Bidmead and Levy (1958)[60] was employed. 10 mls of 30% hydrogen peroxide (100 vol) was mixed with 190 mls of formic acid (98-100%, analytical grade) and 1 ml of methanol. The mixture was left at room temperature for 2 hours to complete the reaction.



performic acid

The solution was then cooled to -10°C in Drikold/acetone and a little extra methanol added (10 mls) to prevent it freezing. The lysozyme (50 mgs) was dissolved in 2 mls of cold formic acid and 25 mls of the performic acid solution was added to the lysozyme

solution. The reaction was allowed to proceed at -10°C for 2½ hours. The reaction mixture was then diluted with 2-3 times its own volume of ice-cold distilled water and the excess performic acid removed by freeze drying. Performic acid converts cystine to two cysteic acids.



Unfortunately side reactions take place involving tryptophan, tyrosine and methionine. Methionine is converted to its sulphone via the sulphoxide. If the solutions are not chloride free, then the reaction with hydrogen peroxide will yield chlorine with which tyrosine will react to give 3-chlorotyrosine or even 3,5-dichlorotyrosine. Performing the oxidation at -10°C helps to prevent these two derivatives being formed.

Tryptophan is completely destroyed during the oxidation to a variety of products all devoid of any peptide activity.

(ii) Cleland's Reagent and Iodoacetic Acid[58]

The lysozyme sample (50 mgs) was dissolved in 10M urea solution (11 mls). Excess Cleland's reagent (dithiothreitol or threo-1,4-dimercapto-2,3-diol) (5 mgs) was dissolved in 1 ml of the urea solution. The lysozyme solution was carefully bubbled with nitrogen (to expell air) for 10 minutes and then Cleland's reagent added. The reaction mixture was stirred at room temperature for 30 minutes under nitrogen. A slight excess of iodoacetic acid in water (0.8 mg in 1 ml water) was then added and the pH adjusted to 8 with triethylamine. The reaction vessel was kept under reduced lighting conditions for a few minutes whilst the mixture was stirred to prevent the formation of iodine. Finally it was dialyzed for 48 hours against water and then freeze dried.

This is a reduction cleavage of the disulphide bond.

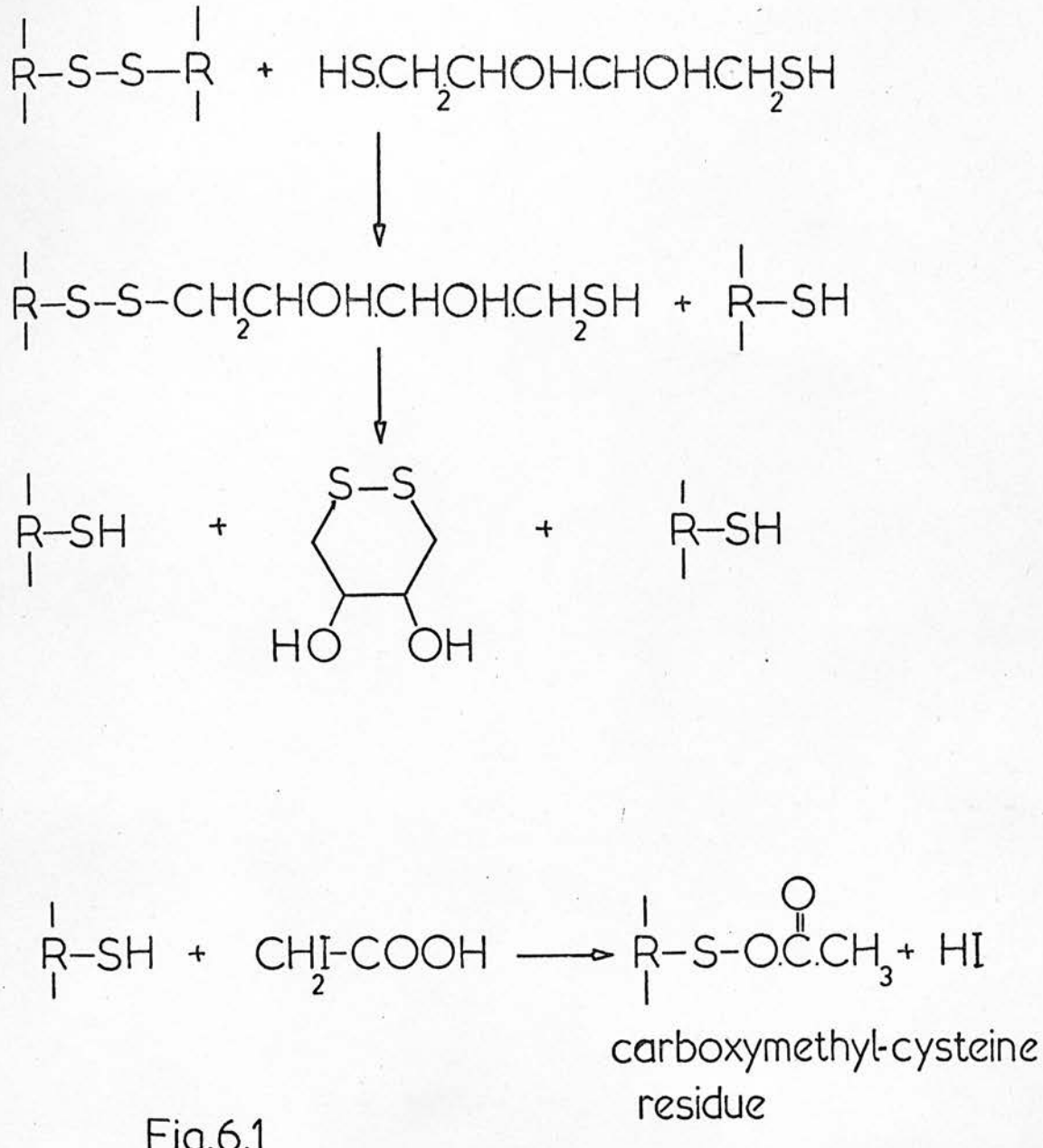


Fig.6.1

Cleland's reagent will also react with iodoacetic acid but much more slowly than cysteine.

The urea acts to break the hydrogen bonds which help to preserve the tertiary structure of the protein. The absence of hydogen bonding makes it easier for the reducing agent to cleave all the disulphide bonds.

Cleland's reagent undergoes no side reactions with the amino acids but excess iodoacetic acid will affect methionine and histidine, and in large excess tyrosine and lysine; methionine undergoes sulphonium salt formation, histidine carboxymethylation and tyrosine and lysine carboxymethylation of their side chains.

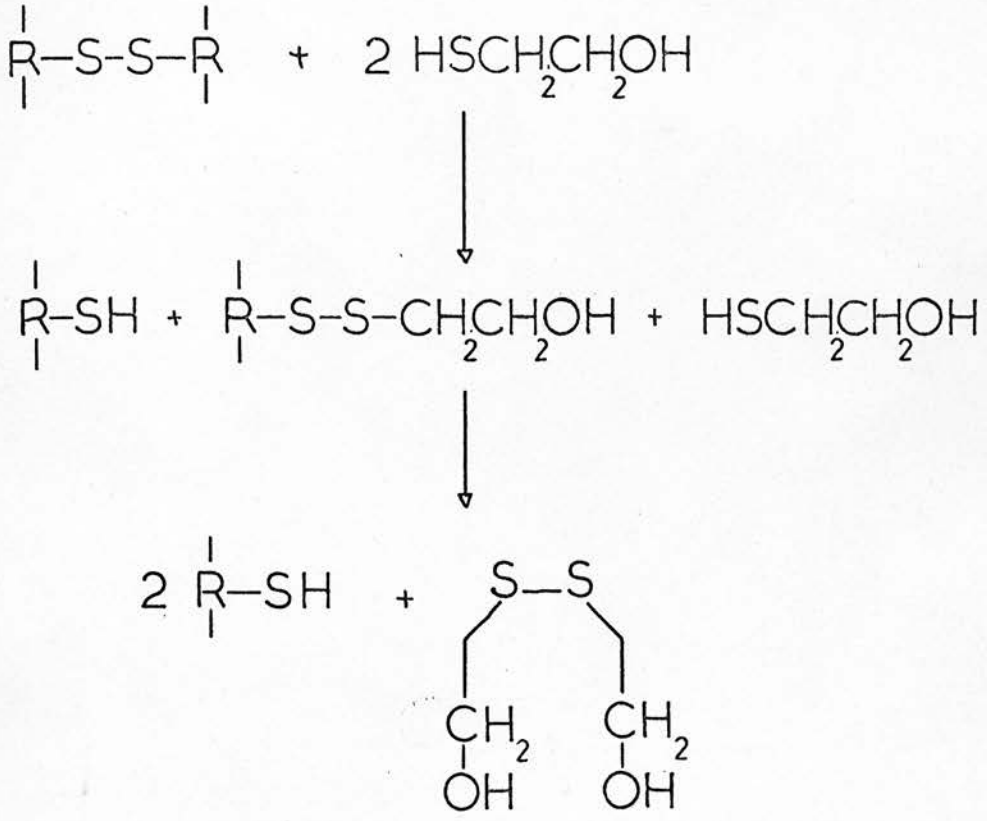
(iii) Mercaptoethanol and Ethyleneimine

The method used was based on that of Raftery and Cole (1963)[61] using the reductive conditions of Crestfield, Moore and Stein (1963)[62]. Concentrated urea solution (calOM) was deionized by passing it through a mixed bed ion-exchange column (42 x 1.25 cms) consisting of equal amounts of Amberlite IR-120 (H^+ form) and Permutit 'Deacidite' FF (OH^- form). This was done to remove contaminating cyanate ions which react with proteins. The effluent was diluted to 9M. with deionized distilled water.

Freshly deionized 9M. urea solution (85 mls) was deoxygenated by bubbling 'spot' nitrogen through it for 20 minutes, lysozyme (450 mgs) was added and dissolved by stirring. To this was added aqueous EDTA solution (4 mls of concentration 50 mgs/ml) and Tris/HCl buffer pH 8.6 (30 mls, 1.44 M in Tris). Tris buffer (and amine buffers generally) minimize the consequences of cyanate ion presence. Nitrogen was carefully bubbled through the stirred solution for 15 minutes taking care that not too much frothing occurred. Excess (2.0 mls.) of 2-mercaptoethanol was added and the solution was stirred at room temperature under nitrogen for 4 hours. Next, ethyleneimine (4 mls) was added and the solution stirred for 2 hours.

The solution was then added to 200 mls of deionized distilled water and dialyzed for 48 hours against water by which time protein had

started to precipitate out of solution. Excess water was removed on the rotary evaporator and then the mixture was freeze dried. The yield is 98-100%. An amino acid analysis was performed on a small sample of the material to check on the amount of β-ae-cysteine present. (See table 6.1). The equations are shown in fig. 6.2.



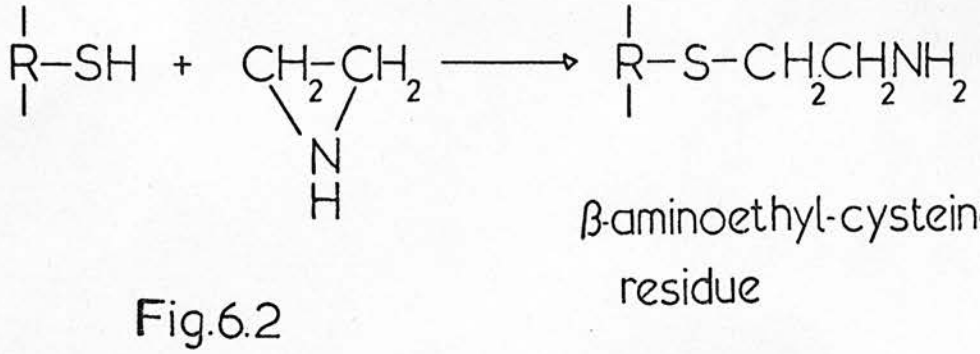


Fig.6.2

The ring of ethyleneimine is opened by the attacking sulphydryl groups. Raftery and Cole have found that the ethyleneimine reaction is quantitative and specific for sulphydryl groups under the conditions used.

2-mercaptoethanol does not affect the other amino acids and ethyleneimine is more satisfactory than iodoacetic acid since it has a greater selectivity for sulphydryl groups and an excess can be used without serious interference from side reactions.

Because of its structural similarity to lysine, ae-cysteine will be cleaved by trypsin. This means that more and smaller peptides will be produced than if the carboxymethyl blocking group had been used.

(b) Preparation of Bovine Globin α-chain and βAe-β-Chain

The adult bovine haemoglobin was a twice recrystallised sample obtained from Miles-Seravac (Berks). It was dehaemed and the α-and β-chains separated. The α-chain contains no cystine and so can be digested in this form. The β-chain however possesses one cystine unit and so the disulphide bond must be broken and the sulphydryl groups blocked.

Two dehaeming procedures were tried:-

(i) Ethyl Methyl Ketone Proceedure

This was performed according to the method of Teale (1959)[63]. Haemoglobin (50 mgs) was dissolved in dilute aqueous hydrochloric acid (10 mls, 0.0375M, pH2) with stirring. To the salt-free solution was added 20 mls of hydrogen peroxide (20 vol.) and the mixture stirred at room temperature for 30 minutes. This oxidizes the iron to the ferric form (metheglobin). The solution was then transferred to a separating

funnel and shaken with 2 x 30 mls of redistilled ethyl methyl ketone. The ketone layer contains the porphyrin ring system (cleaved from the chain by the acid) and the aqueous layer contains the globin chains. The aqueous layer was dialyzed against water and freeze dried. The purity of the globin was checked by running UV and visible spectra of the sample. It was observed that haemoglobin in water gave peaks at 205, 275 and 403 nm; haem in ethylmethyl ketone gave peaks at 240, 359, 450 and 530 nm whilst globin in water produced a spectrum with peaks at 205 and 260 nm. In the haemoglobin spectrum it would appear that the 205 and 260-275 nm peaks represent the absorption due to the globin chains and the 403 nm peak represents the porphyrin ring system. Thus, if the sample of globin is devoid of a peak around 403 nm, then the cleavage and separation of haem has been completed.

Haem, or haem breakdown products, tended to colour the globin a pale brown. This colouration was noticed in the fraction collector samples representing the void volume of the carboxymethyl-cellulose column used in the separation of the globin chains (see later).

(ii) Acid/Acetone Procedure

This was performed according to the method of Fanelli et al (1954)[64]. Haemoglobin (50 mgs) was dissolved in cold water to give a 1-3% solution and this was added dropwise to 100 mls of acetone (analytical grade) at -20°C containing aqueous hydrochloric acid (0.3 mls, 2M). The solution was kept at -20°C for 15-20 minutes with occasional stirring. A white precipitate was formed and this was centrifuged at -20°C for 15 minutes at 2000 r.p.m. (1500 g) using an MSE (London) refrigerated centrifuge. The haem group dissolved in the acetone and was removed, and the globin precipitate was washed twice with cold acetone.

The globin was finally dissolved in cold water (20 mls) and dialysed against water for 16 hours. The resulting suspension was freeze dried to give a fluffy white solid. A UV spectrum was run on an aqueous solution of the solid.

Although this second method is slightly more involved, it was found to give a whiter colour of globin and is probably safer, since the low temperature should prevent any peptide bond cleavage by the acid.

The number of components in this globin sample was ascertained by using disc electrophoresis (see Section 5).

It was now necessary to separate the α-chain from the β-chain in the combined globin samples. The method used was based on that of Labie, Schroeder and Huisman[65] from original work by Clegg, Naughton and Weatherall[66]. The method uses a carboxymethyl-cellulose column and developers of 8M urea containing 0.05 M mercaptoethanol, sodium phosphate buffer and phosphoric acid to a pH of 6.7; a sodium ion gradient was employed from 0.006 M to 0.035 M. The urea was freshly deionized by passing it through a mixed bed resin for each batch of buffer and fresh buffers were made up for each run.

Whatman CM11 carboxymethyl-cellulose powder (4.8 gms, 0.6 m.equiv/gm) was suspended in 300 mls of a solution 0.5 M in sodium chloride and 0.5 M in sodium hydroxide to activate the cation exchanger[67]. It was then filtered, washed thoroughly with water, and suspended in 300 mls of starting buffer. After three removals of fines by allowing the resin to settle for periods of 20 minutes, the resin was resuspended in 200 mls of starting buffer and the column packed under atmospheric pressure. It was packed in layers at room temperature. It was finally equilibrated with starting buffer until effluent pH was the

same as input pH using a flow rate of 40 mls per hour. Equilibration of the column was essential but surprisingly erratic and could take up to several hours. Fresh CM-cellulose was used for each run since Labie et al found that attempted regeneration of used columns resulted in unsatisfactory columns with very high back pressures. They also found that if the resin was passed through a cycle of acid and base before use then the resulting columns were again unsatisfactory.

Work in this laboratory has shown that attempted separations with non-activated resin are unsuccessful. Attempted reseparation of material eluted in the void volumes using a more dilute starting buffer was shown to give no useful fractions; most of the material was eluted in the void volume again.

The globin sample (75 mgs) was dissolved in the minimum of start buffer (ca 1-2 mls, 1-2 hrs.). It was then dialyzed against 3 changes of a twenty fold excess of starting buffer for 3 hours and finally loaded onto the column.

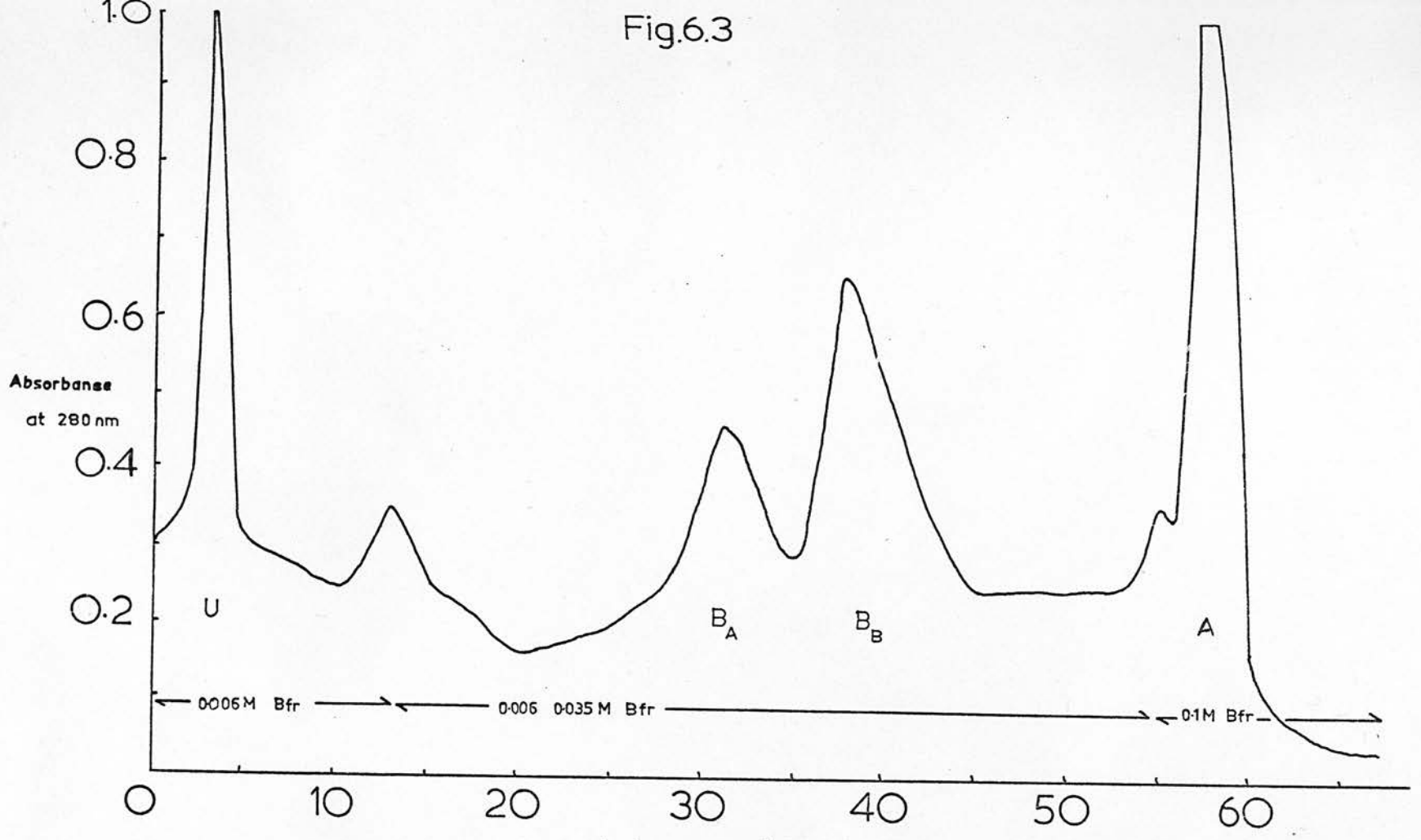
The columns were run at room temperature using a flow rate of 40 mls per hour delivered by a peristaltic pump (LKB ReCyChrom). Starting buffer (50 mls) was pumped through the column and then a sodium ion gradient (2 x 100 mls, 0.006 M to 0.035 M) was used. A stepwise buffer change to a high concentration sodium ion buffer (up to 100 mls, 0.1 M in sodium) was finally employed. Five ml fractions were collected using an LKB Fraction Collector, Distributor and Rotator. Labie et al[65] noted that although the pH should remain constant throughout the run, it was found to vary between 6.5 and 7.0 in the earlier part of the chromatogram before reverting to 6.7. β-chain emerged after the pH had stabilized.

The fractions were analyzed for protein content by measuring the UV absorption at 280 nm. The amino acids responsible for this absorption, tryptophan, phenylalanine, histidine and tyrosine are present in the polypeptide chains. Graphs were drawn of 280 nm absorption against tube number. The contents of tubes of a given peak were pooled, dialyzed against water and then freeze dried. The composition of each peak was ascertained by performing an acid hydrolysis and amino acid analysis on a sample of the material and comparing this with the known composition of α- and β-chains. The type of chain and degree of purity was obtained from these analyses. The α-chain fractions were collected together and stored in a desiccator. The β-chain fractions were also pooled and treated with 2-mercaptoethanol and ethyleneimine to form the β-ae-cysteine derivative as detailed for lysozyme.

All 3 polypeptides were stored in a desiccator whilst awaiting tryptic digestion.

Nine separation runs were performed. Figure 6.3 shows a typical separation pattern. 'U' represents unretarded material i.e. material passing through the column solely under the action of the starting buffer. It was pale yellow-brown in colour and was composed of haem breakdown products and miscellaneous peptide material. The peaks B1 and B2 represent the β-chain and peak A the α-chain. (See Table 6.2). Since the peptide of primary interest to this study was identical for the β_A and β_B chains, the two fractions corresponding to these two peaks were pooled in all runs except for the first two. Here, they were collected separately and analyzed separately. It was found that the first peak corresponded to the β_A chain and the second peak to the β_B chain (see table 6.3). This particular batch of β-chains was not particularly pure.

Fig.6.3



Separation of globin chains on CM-Cellulose cation exchanger

Table 6.1

Amino Acid Analysis of a Sample of β-ae-Lysozyme

Amino Acid Residue	μmoles	Residue Nº	Lit.Value
Asp	0.2284	19.1	21
Thr	0.0769	6.6	8
Ser	0.1062	9.1	9
Glu	0.0593	5.1	5
Pro	0.0201	1.7	2
Gly	0.1361	11.6	12
Ala	0.1231	10.5	12
Val	0.0536	4.6	6
Met	0.0141	1.2	2
Ile	0.0543	4.6	6
Leu	0.0818	7.0	8
Tyr	0.0364	3.1	3
Phe	0.0362	3.1	3
Lys	0.0702	6.0	6
β-Ae-Cys	0.1104	9.4	8
His	0.0145	1.2	1
Arg	0.1184	10.1	11
Trp	-	-	6

Table 6.2

Amino Acid Analysis of bovine Globin Chain fractions from a Carboxymethyl Cellulose Column

Amino Acid Residue	U μmoles	U integer ratio	B1+B2 μmoles	B1+B2 residues	A μmoles	A residues	βchain[57] residues	α-chain[68] residues
Asp	0.0719	14	0.0802	18.5	0.0639	13.3	16(17)(i)	11
Thr	0.0284	6	0.0286	6.6	0.0374	7.8	6	8
Ser	0.0347	7	0.0297	6.9	0.0560	11.7	5(6)	13
Glu	0.0482	10	0.0525	12.1	0.0378	7.9	11	6
Pro	0.0246	5	0.0151	3.5	0.0234	4.9	4	6
Gly	0.0316	6	0.0528	12.2	0.0529	11.0	11(10)	9
Ala	0.0409	8	0.0715	16.5	0.0950	19.8	16	20
½Cys	0.000	0	0.000	0	0.000	0	1	0
Val	0.0312	6	0.0615	14.2	0.0551	11.5	18	12
Met	0.0036	1	0.0064	1.5	0.0016	0.33	3	1
Ile	0.000	0	0.000	0	0.000	0	0	0
Leu	0.0396	8	0.0727	16.8	0.0878	18.3	17	20
Tyr	0.0015	0	0.0079	1.8	0.0124	2.5	2	3
Phe	0.0217	4	0.0442	10.2	0.0362	7.5	10	7

Table 6.2 (Contd.)

Amino Acid Analysis of bovine Globin Chain fractions from a Carboxymethyl Cellulose Column

Amino Acid Residue	U μmoles	U integer ratio	B1+B2 μmoles	B1+B2 residues	A μmoles	A residues	β-chain[57] residues	α-chain[68] residues
Lys	0.0207	4	0.0586	13.5	0.0520	10.8	13(11)	11
His	0.0099	2	0.0284	6.5	0.0490	10.2	6(7)	10
Arg	0.0121	2	0.0207	4.8	0.0168	3.5	4	3
Trp	-		-	-	-	-	2	1

(i) Figure in brackets refers to β_B chain if different to β_B chain

Table 6.3

Amino Acid Analysis Comparison of the two β-chain Fractions from a Carboxymethyl-Cellulose Separation

Amino Acid Residue	B1 μ-moles	B1 residues	B2 μ-moles	B2 residues	β_A-chain[57] residues	β_B-chain[57] residues
Asp	0.1126	19.5	0.0883	18.0	16	17
Ser	0.0437	7.7	0.0386	7.9	5	6
Gly	0.0777	13.5	0.0538	11.0	11	10
Lys	0.0676	11.2	0.0582	11.9	13	11
His	0.0257	4.5	0.0301	6.1	6	7

Section 7

Enzyme Digests

(a) Trypsin and Chymotrypsin Assays

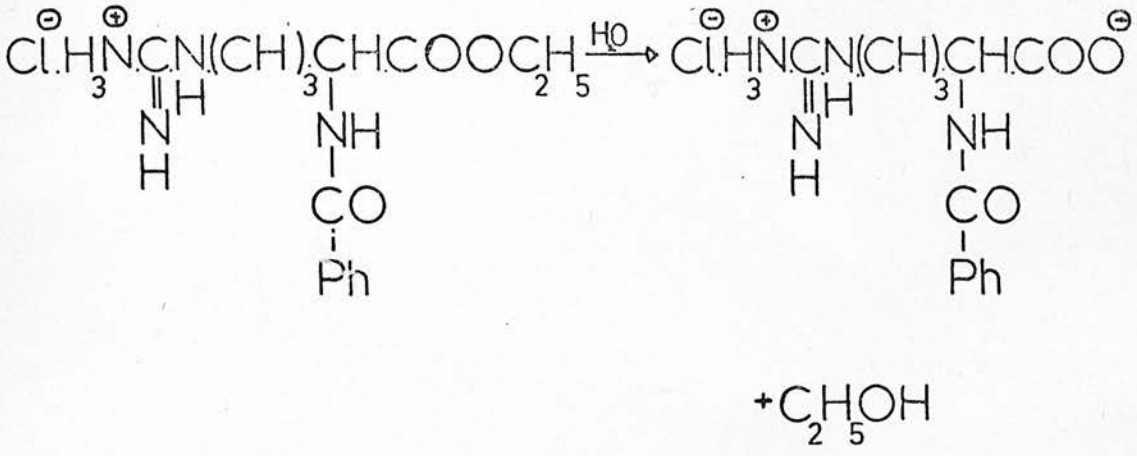
The samples of trypsin were tested for both tryptic and chymotryptic activity. Trypsin samples from BDH Biochemicals and Miles Seravac were tested.

A sample of BDH Biochemicals trypsin was tested for activity using casein as a substrate. One gram of casein was dissolved in 100 mls of 0.1 M Sorensen phosphate buffer[69] pH 7.6 by heating to boiling for 15 minutes. This 1% solution was kept in a cool place until required; it is stable for about 1 week. The trypsin was dissolved in aqueous hydrochloric acid (0.0025 M) to a concentration of 0.02 mg per ml. and dilutions made using this acid.

One ml samples of trypsin solution from 0.001 mg/ml to 0.2 mg/ml in concentration were mixed with 1 ml samples of the casein solution and the mixtures incubated at 37°C for 20 minutes. 3 mls of 5% trichloroacetic acid were then added to precipitate any undigested protein. The samples were left at room temperature for 1 hour and then centrifuged for 15 minutes at 3,800 g. The supernatents were removed and the optical density at 280 nm measured. A graph of optical density against enzyme concentration was plotted. Controls were run using maximum enzyme and no enzyme, with prior precipitation of the casein by trichloroacetic acid in the former case. Casein it should be noted contains calcium ions which act to stabilize the enzyme against autodigestion.

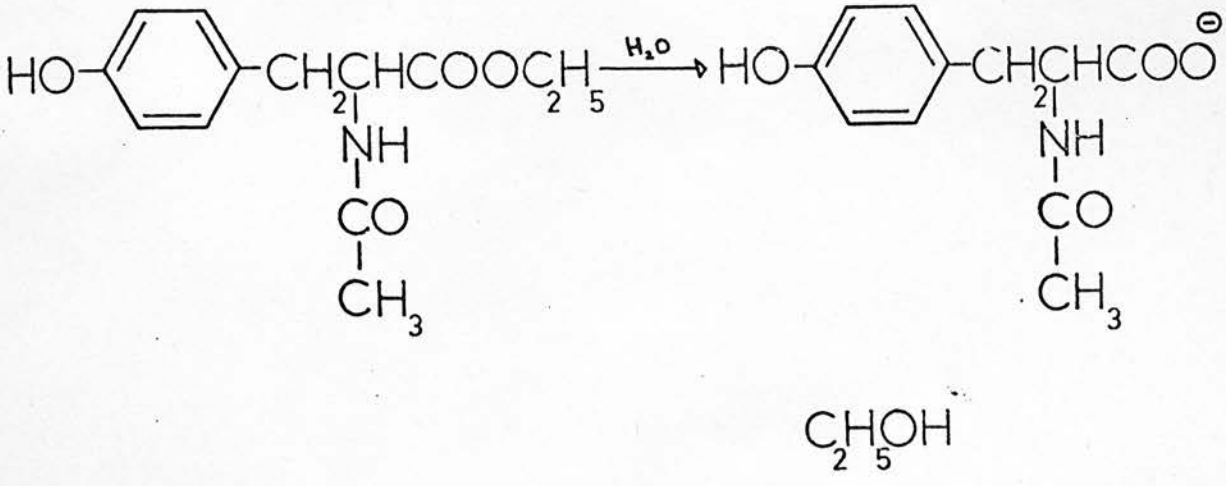
Both the trypsin samples were tested by using the standard trypsin assay method of Schwert and Takenaka[70], except that the concentration of substrate was a half that given in the text. The substrate N-benzyl-arginine ethyl ester hydrochloride was used and the change

in optical density at 253 nm was measured against time.



A change in absorption at 253 nm is a measure of the ionization of the carboxyl group of the arginine derivative, absorption increases with increase in tryptic activity.

The trypsin samples were also tested for their chymotryptic activity along with a sample of chymotrypsin, again using a method devised by Schwert and Takenaka (1955)[70]. Since a sample of N-acetyl-L-tyrosine was not available with which to zero the SP500 UV spectrometer, the substrate was zeroed at 237 nm by means of the slit width at an OD value of 0.500. The substrate N-acetyl-L-tyrosine ethyl ester was used and the decrease in optical density at 237 nm with time was measured.



The change in OD at 237 nm is due to the change in ionization of the carboxyl group of the substrate. The use of N-acetyl-tyrosine ethyl ester as a chymotryptic substrate is complicated by two tendencies of opposing influence which arise as the pH is increased beyond the range of dissociation of the carboxyl group of N-acetyl-tyrosine. Firstly the spectral difference between the carboxylic acid and the carboxylate form of acetyl-tyrosine tends to be obscured by the large absorption due to the ionization of the phenolic hydroxyl group. Secondly, the enzyme activity increases with pH, becoming maximal at pH 8.2. As a compromise between the two opposing effects, a pH of 7.0 in 0.05 M phosphate buffer is used.

The Miles-Seravac trypsin was treated with diphenyl-carbamyl chloride to inhibit its small amount of chymotryptic activity. The DPCC treatment was carried out in this laboratory using the method of Erlanger and Cohen (1963)[71].

It was found that both the samples of trypsin were tryptically active and to a very much lesser extent chymotryptically active. Trypsin will, however, attack the chymotryptic assay substrate but at a very much reduced rate compared with chymotrypsin. The results are shown in table 7.1. Although these results indicated a very low level of chymotryptic activity, prior study of these enzymes with bovine α-chain globin indicated that digestion other than tryptic was taking place as well as the expected tryptic digestion. Possibly this secondary digestion is of the 'pseudotryptic' type.[72] Pseudotrypsin shows good activity with ATEE but reacts only poorly with BAEE. α-trypsin[72] is converted to Q-trypsin under the same conditions as for the tryptic cleavage of protein and it is possible that we are getting this form

of trypsin produced. Its cleavage action is slow and incomplete, but has been found to act on the carboxyl groups of phenylalanine tyrosine, tryptophan and even asparagine.

The BDH trypsin activity was confirmed by the digestion with casein.

The DPCC treated trypsin was no less chymotryptically active than the untreated material and it has lost some of its tryptic action with reference to the assay substrates. It was however decided to try it out with a sample of lysozyme or globin α-or β-chain. The BDH trypsin was also used in the natural substrate digestions.

Table 7.1(a)

Tryptic Activity of Samples of Trypsin

Trypsin Sample	Enzyme Conc. (mg/ml)	Rate of Increase in OD w.r.t. time (units per min)
BDH	0.0073	0.200
Miles-Servac	0.0073	0.129

One unit of tryptic activity is that amount of enzyme which causes an increase in the optical density of 0.001 unit per minute under the assay conditions.

$$\text{BDH trypsin activity} = \frac{0.200}{0.001} \times \frac{1}{0.0073 \times 3} \text{ BAEE units per minute}$$

$$= 9{,}100 \text{ BAEE units per minute}$$

Miles-Seravac trypsin activity = 5,870 BAEE units per minute

Specific Activity[70] is approximately 9,000 units per minute

Table 7.1(b)

Chymotryptic Activity of Samples of Trypsin and Chymotrypsin

Trypsin Sample	Chymotrypsin	BDH	Miles-Seravac
Enzyme Concentrations (mg per ml)	0.067	0.067	0.067
Rate of increase in OD w.s.t. time (units per minute)	0.035	0.0067	0.0055

One unit of chymotryptic activity is that amount of enzyme which causes a decrease in the optical density of 0.001 unit per minute under the assay conditions.

Chymotryptic activity of BDH trypsin = 33 ATEE units per minute

" " " MS trypsin = 27 ATEE units per minute

" " " Chymotrypsin sample = 1,740 ATEE units per minute

Specific Activity of Chymotrypsin is approximately 10,000 units per minute

Table 7.1(c)

Tryptic and Chymotryptic Activity of DPCC treated and untreated Trypsin

Trypsin Sample	MS trypsin	DPCC treated MS trypsin
Tryptic Activity (BAEE units/min.)	5,870	4,000
Chymotryptic Activity (ATEE units/min.)	27	31

(b) Tryptic Digests of βae-lysozyme and βae-bovine globin β-chain

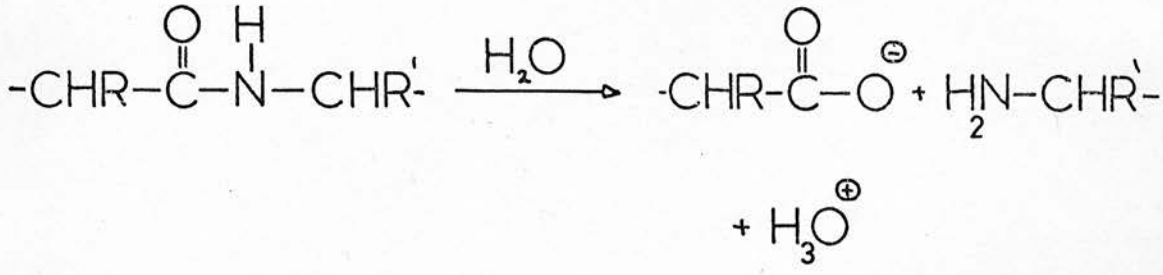
Tryptic digestions were carried out using a Radiometer (Copenhagen) titrigraph (SBR2C) as a pH-stat, a Radiometer syringe burette (SBU1a) and a Radiometer titrator (TTTIC).

The modified protein was dissolved in distilled water containing a little acetic acid, to a concentration of 2%. The mixture was warmed to 37°C and nitrogen was gently bubbled through the solution for 20 minutes to remove air and carbon dioxide. The nitrogen tube was raised above the level of the liquid and then aqueous sodium hydroxide solution (0.1 M or 0.5 M) added from the burette until the pH was 7.9. This reagent was found to be more suitable than aqueous ammonium carbonate solution (2M) or aqueous ammonium bicarbonate solution (2M), both with initial pH adjustment by aqueous ammonium hydroxide solution (2M). These reagents increased the quantity of salt in the mixture which made freeze drying more difficult. At pH 7.9 it was found that most of the protein precipitated out of solution. A little DPCC treated trypsin, or BDH trypsin, was dissolved in 2 drops of an aqueous solution 0.001 M in hydrochloric acid and 0.01 M in calcium chloride, and this was added to the protein suspension. The pH-stat and recorder were switched on, and the digestion was performed at 37°C under nitrogen. Two or three more samples of trypsin were added to give a final enzyme to substrate ratio of not more than 1:75.

After 24 hours, or less if the recorder trace indicated that the reaction had gone to completion, the digestion was stopped. Glacial acetic acid was added to a pH of 5. The peptide mixture was centrifuged if necessary and freeze dried along with any precipitate washings. Any solid protein was freeze dried separately. The small

amount of salt present did not interfere with the freeze drying process. The freeze dried peptide mixture was kept in a dry state until required for separation.

Trypsin cleaves peptide bonds involving the carboxyl groups of lysine, arginine and, more slowly, aminoethyl-cysteine to produce peptides that are C-terminal in these residues. Chymotrypsin is less selective than trypsin and will cleave peptide bonds involving the carboxyl groups of tryptophan, tyrosine, phenylalanine and leucine and more slowly others including methionine, isoleucine, valine and histidine. Each cleavage releases a hydrogen ion into solution and so the solution would become progressively more acidic. Since trypsin is only active in the pH range 7-9 with an optimum pH of 7.8-8.2, then it is desirable to keep the pH constant. With the pH-stat, hydroxyl ions were released into the solution to neutralize the hydroxonium ions formed by the digestion.



From each peptide bond cleavage one hydroxonium ion is produced and so one hydroxyl ion is required to restore the pH to its preset value. Thus a measure of the degree of enzyme digestion can be ascertained from the amount of alkali added.

The example of the lysozyme (1 gm) digestion will be considered.

M.Wt. of lysozyme = 14,500

Wt. of lysozyme employed = 1.0 gms.

Let the number of enzyme cleavage points per chain be x.

Nº of moles of lysozyme used $= \frac{1.0}{14,500}$

Nº of chains in this quantity of material $= \frac{1.0}{14,500} \times A$ where A is Avagadros Nº.

Nº of cleavage points in the sample $= \frac{1}{14,500} \times A \times X$

and this is also equal to the number of hydroxonium ions produced.

Molarity of the aqueous sodium hydroxide solution used = 0.5 M

No. of mls of this solution used = 4.8 mls.

The influence of the small amount (ca 0.2 mls) of the 0.001 M aqueous hydrochloric acid solvent for the enzyme will be neglected. Nº of moles of alkali added to the digestion mixture $= \frac{4.8}{1000} \times 0.5$

No. of hydroxyl ions added to the digestion mixture $= \frac{4.8}{1000} \times 0.5 \times A$

If the pH is to remain static then

$$\frac{1}{14,500} \times A \times X \text{ must equal } \frac{4.8}{1000} \times 0.5 \times A$$

$$X = 14,500 \times \frac{4.8}{1000} \times 0.5$$

$$= 34.75 \text{ moles (OH)/mole lysozyme}$$

$$= 35 \text{ since X must be an integer.}$$

No. of tryptic cleavage points in the lysozyme chain = 25.

The tryptic peptides for βae-lysozyme and those for βae-bovine globin β-chain are given in tables 7.2 and 7.3 respectively.

Some chymotryptic peptides were produced but this was not too disadvantageous to this work since, although a more complex peptide mixture was produced, the peptides were often smaller than they would have been and so easier to use.

Trypsin does not split all potential points of cleavage with equal speed or success e.g. the rate of cleaving a Lys-Pro or Arg-Pro bond is nearly zero; the rate is greatly reduced with Asp-Arg or Asp-Lys (note Globin β_{AB} T12). Also if the potential point of cleavage is near the N or C terminus of a chain then trypsin is slowed or stopped in its action.[96]

Table 7.2

Tryptic Peptides of β-Ae-lysozyme[23]

T1:- Lys

T2:- Val-Phe-Gly-Arg

T3:- βAe-Cys

T4:- Glu-Leu-Ala-Ala-Met-Lys

T5:- Arg

T6:- His-Gly-Leu-Asp-Asn-Tyr-Arg

T7:- Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-β-Ae-Cys

T8:- Ala-Ala-Lys

T9:- Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg

T10:- Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Glu-Ile-Asn-Ser-Arg

T11:- Trp-Trp-β-Ae-Cys

T12:- Asn-Asp-Gly-Arg

T13:- Thr-Pro-Gly-Ser-Arg

T14:- Asn-Leu-β-Ae-Cys

T15:- Asn-Ile-Pro-β-Ae-Cys

T16:- Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-β-Ae-Cys

T17:- Ala-Lys

T18:- Lys

T19:- Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg

T20:- Asn-Arg

T21:- β-Ae-Cys

T22:- Lys

T23:- Gly-Thr-Asp-Val-Gln-Ala-Trp-Ile-Arg

T24:- Gly-β-Ae-Cys

T25:- Arg

T26:- Leu

Note:

T5 = T25 = Arg

T1 = T18 = T22 = Lys

T3 = T21 = β-Ae-Cys

Table 7.3

Tryptic Peptides of β-Ae-bovine globin β-chain[57]

β_{AB}-T1:- Met-Leu-Thr-Ala-Glu-Glu-Lys

β_{A}-T2:- Ala-Ala-Val-Thr-Ala-Thr-Trp-Gly-Lys

β_{B}-T2:- Ala-Ala-Val-Thr-Ala-Thr-Trp-Ser-Lys

β_{A}-T3a:- Val-Lys

β_{A}-T3b:- Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-(Leu,Gly)Arg

β_{B}-T3:- Val-His-Val-Asp-Glu-Val-Gly-Gly(Glu,Ala,Leu,Gly)Arg

β_{AB}-T4:- Leu-Leu-Val-Val-Tyr-Pro(Trp,Thr,Gln)Arg

β_{AB}-T5:- Phe-Phe-Glu-Ser-Phe-Gly(Asp,Leu,Ser,Thr,Ala,Asp,Ala,Val,Met, Asp,Asn,Pro)Lys

β_{AB}-T6:- Val-Lys

β_{AB}-T7:- Ala-His-Gly-Lys

β_{AB}-T8:- Lys

β_{AB}-T9:- Val-Leu-Asp-Ser-Phe-Ser(Asp,Gly,Met)-Lys

β_{AB}-T10:- His-Leu-Asp-Asp-Leu-Lys

β_{AB}-T11:- Gly-(Thr,Phe,Ala,Ala,Leu,Ser,Glu,Leu,His,β-Ae-Cys)

β_{AB}-T12:- Asp-lys

β_{AB}-T13:- (Leu,His,Val,Asp,Pro,Glu,Asn,Phe)Lys

β_{B}-T14:- (Leu,Leu,Gly,Asn,Val,Leu,Val,Val,Val,Leu,Ala)Arg

β_{A}-T14:- No peptide

β_{A}-T15a:- Asn-Phe-Gly-Lys

β_{A}-T15b:- (Glu,Phe,Thr,Pro,Asp,Val,Gln,Ala,Leu,Phe,Gln)Lys

β_{B}-T15:- Asn-Phe-Gly-Asn-(Glu,Phe,Thr,Pro,Asp,Val,Gln,Ala,Leu-Phe-Gln) Lys

β_{AB}-T16:- Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg

Table 7.3

Tryptic Peptides of β-Ae-bovine globin β-chain[57]

Contd.

β_A-T17:- (Tyr,His)

β_B-T17:- Tyr-His

Section 8

Peptide Separations and Identification

The tryptic digests were dissolved in the minimum amount of starting buffer (0.05 M pyridine formate, pH 2.5) and loaded onto the Zeo-Karb 225 8% cation exchange preparative column. The peptides were separated using a sigmoid gradient followed by a linear one (except for the Lyl separation where two linear gradients were used). The fractions were collected using the LKB fraction collector described in Section 6. Tables 8.1 & 2 give full details of the separations.

Alternate tubes were tested for peptide content by means of the peptide analyzer. On the basis of the separation trace produced by the analyzer, tubes were pooled. Initially the pooled fractions were concentrated on the rotary evaporator at <40°C before being air dried and finally vacuum dried. Latterly they were freeze dried, re-dissolved in a little pyridine acetate buffer (1.0 M, pH 4.5) and then air and vacuum dried over phosphorus pentoxide. No samples were discarded in this first separation.

From fractions corresponding to the less complex peaks, samples of ca $\frac{1}{20}$" of the available material were taken before the air drying stage, and analyzed for amino acid content. The peptides of most interest to this study will probably be contained in the fractions derived from the first half of the first gradient. Fractions which, on the basis of their amino acid analysis, might be of interest were re-separated individually on the Amberlite 200 preparative column. Samples corresponding to complex peaks were also refractionated on Amberlite 200. The peptide analyzer was used to monitor the separation and pooled fractions were dried as before. Samples corresponding to all peaks were

taken and analyzed for amino acid composition.

If considered necessary the sample was separated on a third column, the anion exchanger "Deacidite" FFIP.

The gradient employed for the separations on Amberlite 200 varied from being concave to convex in molarity depending on the fractions to be reseparated. The concave gradients - the ones most often used - have been shown to be linear in pH[50]. Normally a 3-chamber system was used and the molarity of buffer at any given stage of the separation was calculated by the method of Boch and Ling.[73] For a 2-chamber system:-

$$C_{col} = C_{2\rightarrow 1} - (C_{2\rightarrow 1} - C_1)(1-V)^{A2/A1}$$

Where C_{col} = molarity of buffer entering the column

$C_{2\rightarrow 1}$ = " " " " Chamber 1

C_1 = " " " in " 1

V = Fraction of gradient (0 to 1)

A_1 = Cross-sectional area of Chamber 1

A_2 = " " " " " 2

In this lab. $A_1 = A_2$

For a 3-chamber system:-

$$C_{2\rightarrow 1} = C_{3\rightarrow 2} - (C_{3\rightarrow 2} - C_{2\rightarrow 1})(1 - V)$$

A stepwise gradient change was used for the anion exchanger.

The tryptic peptide in β-chain globin of most interest to this study is β-T9. This peptide contains methionine next to the Asp-Gly sequence under observation. After separation of the tryptic digest on Zeo-Karb 225 8% it was decided to test all the fractions for the presence of methionine.

The peptides were dissolved in pyridine acetate buffer (0.5 ml, 1.0 M, pH 4.5) and small duplicate samples spotted onto Whatman No.1 chromatography paper and dried. One spot was sprayed with ninhydrin

solution (0.2% in 90% aqueous acetone) and warmed to develop the colour. The other spot was sprayed with an aqueous solution of potassium dichromate (0.1 M) and glacial acetic acid (1:1 ratio)[74]. After drying, it was sprayed with aqueous silver nitrate solution (0.1M). Methionine is indicated by an orange spot against a red background. This test was performed on both β-chain separations and the results are given in table 8.3.

Thin layer electrophoretic separations of the 'likely' peptides from the results of the methionine test and from the literature were run. The results are shown in table 8.4. The calculation of Bock and Ling[73] was used to ascertain the molarity of buffer needed to elute βT9 from the literature system. The fraction corresponding to this molarity on our (comparable) system was determined. It was found to agree with the methionine results, i.e. in the fraction range βT1I,J and βT2G,H,I.

Table 8.1

Preparative Column Fractionation Details

	First Column	Second Column	Third Column
Resin	Zeo-Karb 225 8%	Amberlite 200	Deacidite FFIP
Type of Exchanger	Cation	Cation	Anion
Column Dimensions	105 x 1.25 cms	91 x 1.25 cms	38.5 x 1.20 cms
Jacket temperature	50°C	50°C	37°C
Buffers	pyridine formate pyridine acetate	pyridine formate pyridine acetate	See Table 8.2
Buffer pH gradient	(i)2.5 - 5.0 (ii)5.0 - 5.0	3.1 - 5.0	See Table 8.2
Buffer base Concentration Gradient	(i)0.05M-2.0M (ii)2.0M- 5.0M	0.1M-2.0M (- 5.0M)	Stepwise change
Typical buffer volume	(i)1200mls (ii) 600 mls	600 mls	688 mls
Buffer flow rate	24 mls/h	24 mls/h	24 mls/h
Buffer regenerant	Starting buffer	Starting buffer	Starting buffer
Back pressure	20-50 p.s.i.	80-160 p.s.i.	atmospheric
Fraction size	2.8 mls	2.8 mls	2.8 mls

Table 8.2

Anion Exchange Buffers

			Amount Used
1.	α-picoline (0.90M) N-ethylmorpholine (0.45M) pyridine (0.52M)	) plus acetic acid to pH 9.4	- 24 mls
2.	"	) plus acetic acid to pH 8.6	- 48 mls
3.	"	) plus acetic acid to pH 6.5	- 96 mls
4.	Acetic acid (0.5 M)		- 120 mls
5.	Acetic Acid (2.0 M)		- 200 mls
6.	Formic Acid (2.0 M)		- 200 mls

Stepwise addition of buffers

Table 8.3

Methionine Spot Test Results for β-chain Globin Tryptic Peptides

β-chain 1 peptide			β-chain 2 peptides		
Peptide (βT1)	Ninhydrin result	Methionine result	Peptide (βT2)	Ninhydrin result	Methionine result
A	grey-brown	-	A	grey-purple	-
B	pale purple	±	B	purple	±
C	grey	±	C	light-brown purple	+
D	light purple	-	D	red purple	±
E	light purple	-	E	purple	-
F	purple	-	F	purple	+
G	purple	±	G	red-purple	+
H	purple	+	H	purple	+
I	red-purple	+++	I	purple	+
J	purple	±	J	purple	-
K	red-purple	-	K	purple	-
L	purple	-	L	purple	-
M	brown purple	+	M	brown-purple	++
N	brown purple	-	N	brown-purple	+
O	brown purple	-	O	brown-purple	-
P	light red-purple	++	P	light brown-purple	±
Q	light brown-purple	±	Q	purple	±
R	purple	+	R*	brown	-
S*	grey brown	+			
T*	brown	±	blank	-	-
			Met Control	light brown purple	+++

* peptide solution dark brown in colour.

Key for methionine test:

-	No orange colour
±	Trace of orange colour only
+	Pale orange
++	Deeper orange
+++	Distinctive orange colour

Table 8.4

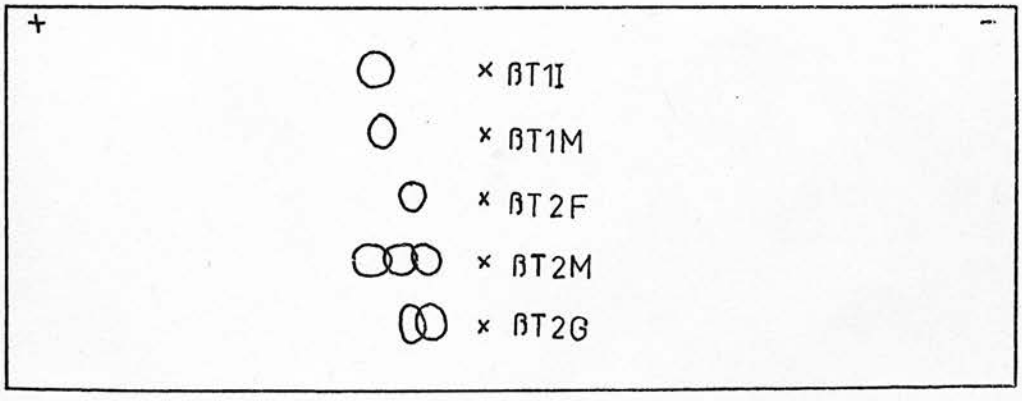
Thin Layer Electrophoresis of β-chain Globin Tryptic Peptides

(i) Stationary phase - MN-Cellulose.

(ii) Buffer - pyridine acetate (1.0 M, pH 6.5).

(iii) Voltage - 300 V - 11 V/cm.

(iv) Time - 60 minutes.



At this pH all the peptides were present as anions.

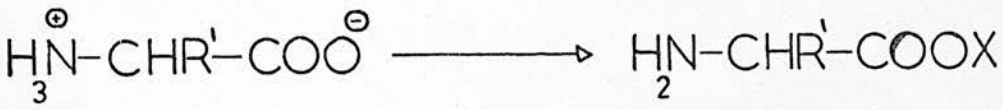
Section 9

Synthetic Work

In its most general form the synthesis of peptides involves the following stages:[75]

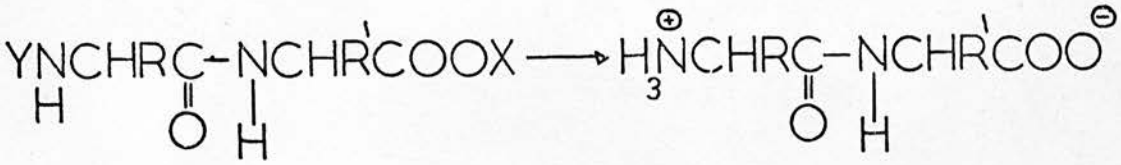
(i) Preparation of a 'carboxyl component' by blocking the amino group of an amino acid or peptide with a group, Y.

(ii) Synthesis of an 'amino component' by protecting the carboxyl group of another amino acid or peptide with a group, X.

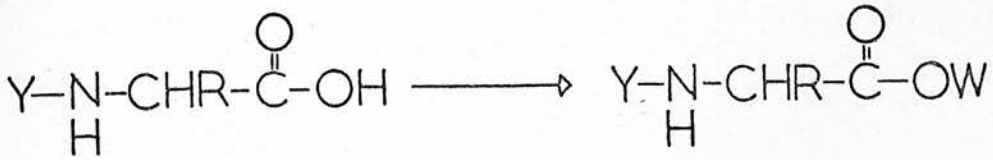


(iii) Activation of the carboxyl group of the 'carboxyl component' (or less commonly the amino group of the 'amino component') with a group W' and coupling of the ensuing intermediate with the amino component (or carboxyl component) to give a protected peptide (fig. 9.1).

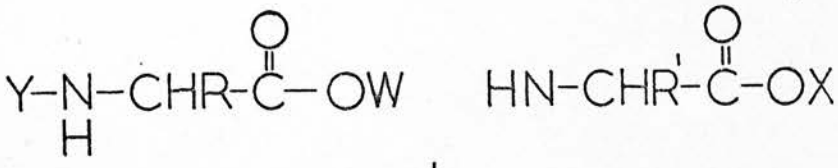
(iv) Removal of the blocking groups Y and Z from the protected intermediate to form the free peptide.

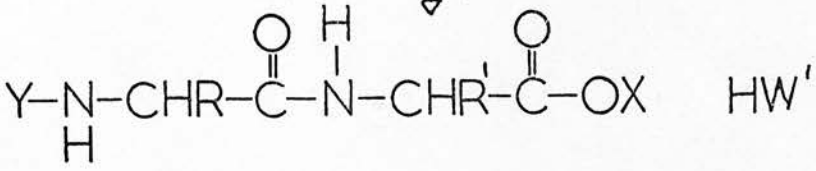


In elongating a peptide chain, one of the blocking groups is removed and the resulting new amino or carboxyl component is processed further as for stage (iii). This is followed by removal of the blocking groups to give the free peptide.

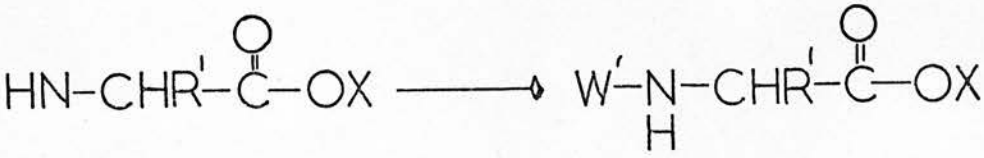


then

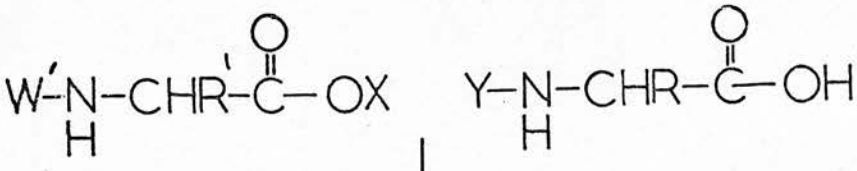




or



then



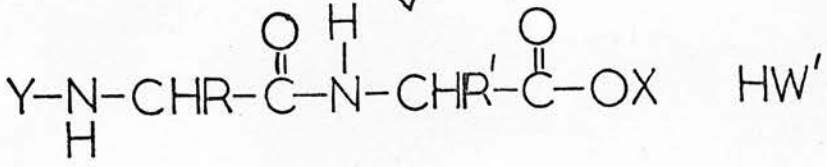


Fig.9.1

The amino and carboxyl protecting groups should satisfy the following criteria:-

(a) They should react readily with the amino/carboxyl group of amino acids or peptides under experimental conditions which do not impair the chemical or steric nature of the rest of the molecule.

(b) They should resist the various manipulations required for synthesis, isolation and purification of peptides.

(c) They should be removable selectively from the final product under conditions which will not affect the rest of the peptide molecule.

Examples are tert-butyl or benzyl esters in the formation of 'amino components' and benzyloxycarbonyl chloride or butyloxycarbonyl chloride in the formation of 'carboxyl components'.

In this work the acetate salt of asparaginyl-glycyl ethyl ester was synthesized using the following reaction scheme:-

1. Z-Asn ⟶ Z-Asn-OSu

2. Z-Asn-OSu + Gly-OEt ⟶ Z-Asn-Gly-OEt

3. Z-Asn-Gly-OEt ⟶ Asn-Gly-OEt

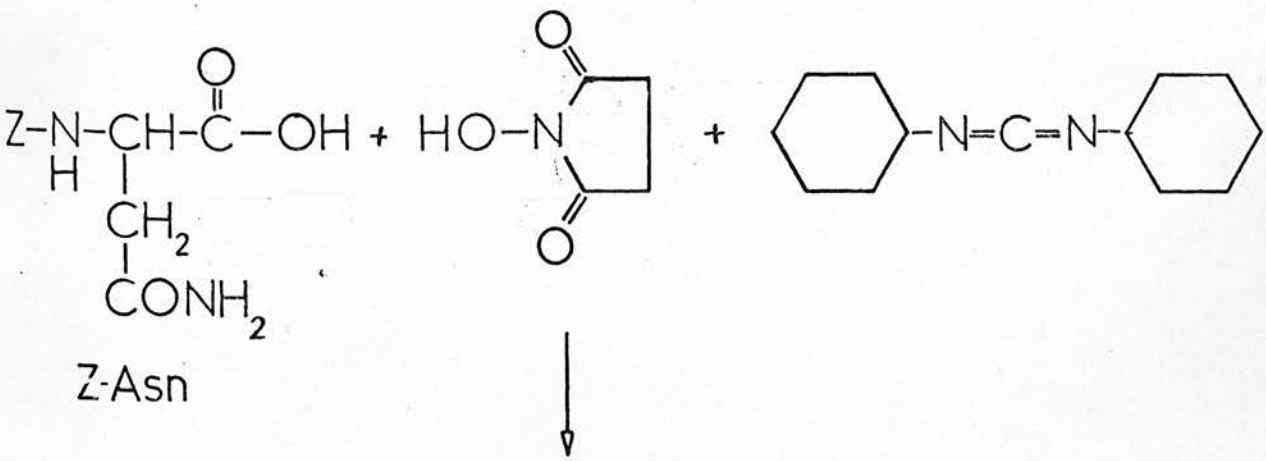
Z-Asparagine was purchased from Sigma (USA) but could have been produced from asparagine and benzyloxycarbonyl chloride[76]. Glycine ethyl ester hydrochloride was available in the laboratory and had been prepared previously by the method of Curtius and Goebel using a suspension of glycine in absolute methanol (or ethanol) and dry hydrogen chloride gas[77]. IR spectra were run on start materials and products and the results are given in table 9.1.

1. Preparation of Z-Asn-OSu from Z-Asn

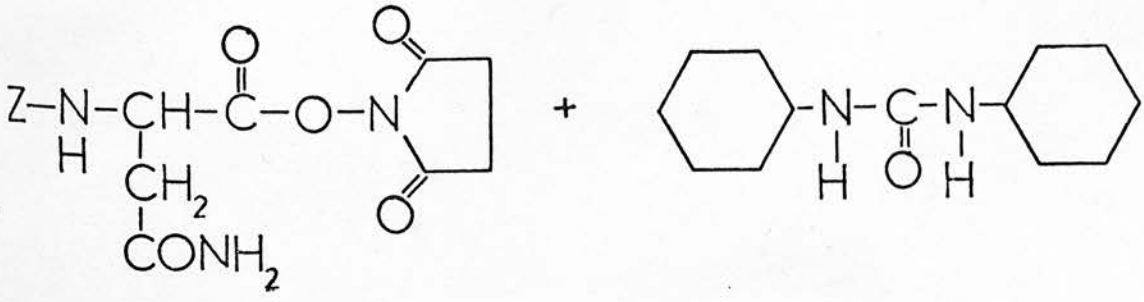
The method used was based on that of Anderson, Callahan and Zimmerman.[78] Benzyloxycarbonyl-asparagine (4 gms, 0.015 moles) was

dissolved by warming in dioxan (60 mls). The solution was cooled and then N-hydroxy succinimide (1.732 gms, 0.015 moles) added and dissolved by stirring; N,N-dicyclohexylcarbodiimide (3.1 gms, 0.015 moles) was added and also dissolved by stirring. The mixture was left overnight at room temperature. The heavy white precipitate of dicyclohexylurea was filtered off and washed with a little dioxan. The filtrate was concentrated on the rotary evaporator and an oily crystalline solid appeared. This was triturated with ether and the solvent decanted away. The solid was redissolved in methylene chloride and then re-precipitated with pet-ether. Since the precipitate was sticky and difficult to filter, the solvents were removed by rotary evaporation and the purification procedure repeated. After final removal of solvent by rotary evaporation the white solid was filtered, washed with ether and finally dried in vacuo.

Yield - 4·1 gms ; % Yield - 76% ; m.pt. - 115°C



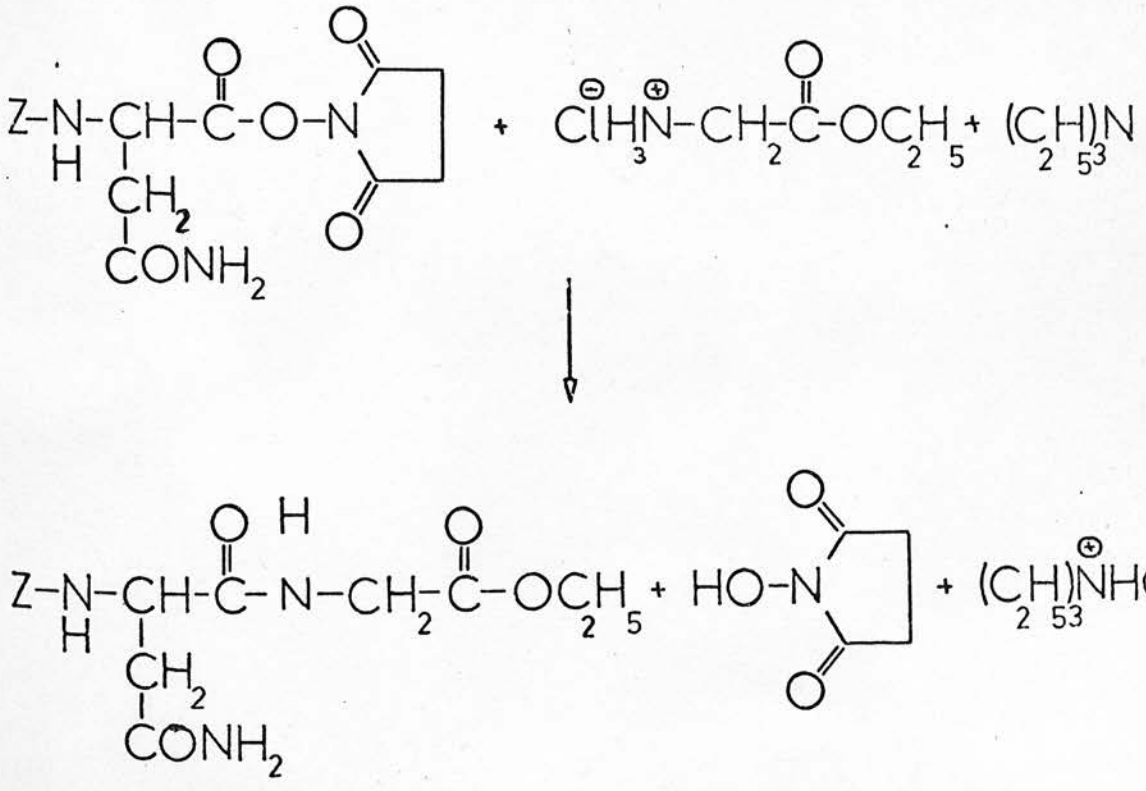
Z-Asn



Z-Asn-O-Su

2. Preparation of Z-Asparaginyl-glycyl ethyl ester

The method was based on that of Anderson, Callahan and Zimmerman[79]. Equimolar quantities of Z-asparaginyl-O-succinimide (3.3 gms), glycine ethyl ester hydrochloride (1.13 gms) and triethylamine (1.23 mls) were employed. The Z-Asn-O-Su was dissolved by stirring in cold dioxan (40 mls) and the triethylamine added. The glycine ethyl ester hydrochloride was dissolved in methanol (100 mls) and then added to the Z-Asn-OSu solution. A slight turbidity was noticed. The mixture was stirred at room temperature for 30 minutes and then filtered to remove the turbidity from the solution. Cold water (30 mls) was added and since no precipitate was produced the solvents were removed in vacuo by the rotary evaporator. The white solid produced was shaken with tepid water (<40°C) to remove N-hydroxy-succininide, triethylamine hydrochloride and unreacted glycyl ester and then filtered. The remaining solid was recrystallized from boiling water. The precipitate was filtered, washed with a little cold water and then dried in a drying pistol.



Z-Asn-Gly-O-Et

Yield of Recrystallized material = 1.3 gms : % yield = 41%

M.pt. of crude material = 167°C

M.pt. of recrystallized material = 172°C

The filtrate from the tepid water extraction was dried on the rotary evaporator under vacuum. A white solid was produced which turned yellow and sticky on heating to 80°C.

3. Preparation of the Acetate Salt of Asparaginyl-glycyl ethyl ester

The reductive action of hydrogen under the catalytic influence of palladium charcoal was utilized[80] to remove the benzyloxycarbonyl blocking group from the N-terminal of asparagine. Z-asparaginyl-glycyl ethyl ester (0.7 gms, 0.0018 moles) was dissolved in warm methanol/dioxan (50 mls and 80 mls) and then cooled. Palladium charcoal (10%, 0.15 gms) was suspended in methanol (10 mls) and glacial acetic acid added (5 drops i.e. >1.2 molar equivalents). The hydrogenation was performed at room temperature and atmospheric pressure for 4 hours with stirring such that the stirrer bar broke the surface of the liquid. A dreschel bottle of dilute barium hydroxide solution was incorporated in the system next to the water pump. After 4 hours the excess hydrogen and waste gases were pumped away and passed through the bottle on their way to the pump. The formation of a white precipitate of barium carbonate indicated the presence of carbon dioxide.

After removal of the hydrogenation flask the catalyst was filtered off (twice) and solvents removed in vacuo on the rotary evaporator as far as possible. A little acetic acid (2 mls) has added and the solvent removed again using the rotary evaporator (temperature kept below 40°C). The mixture was concentrated to a thick oil and then placed in a vacuum desiccator over phosphorus pentoxide for several days. Addition

of methanol gave a pale yellow solution and this was again dried *in vacuo*, some of the oil was removed and dissolved in 2 mls of water and stored in a frozen state. The remainder was stored over phosphorus pentoxide (initially under vacuum) for several months and methanol again added. This time a pale yellow solid appeared and this was filtered, washed with a little cold methanol and vacuum dried to a white crystalline solid (fig. 9.2).

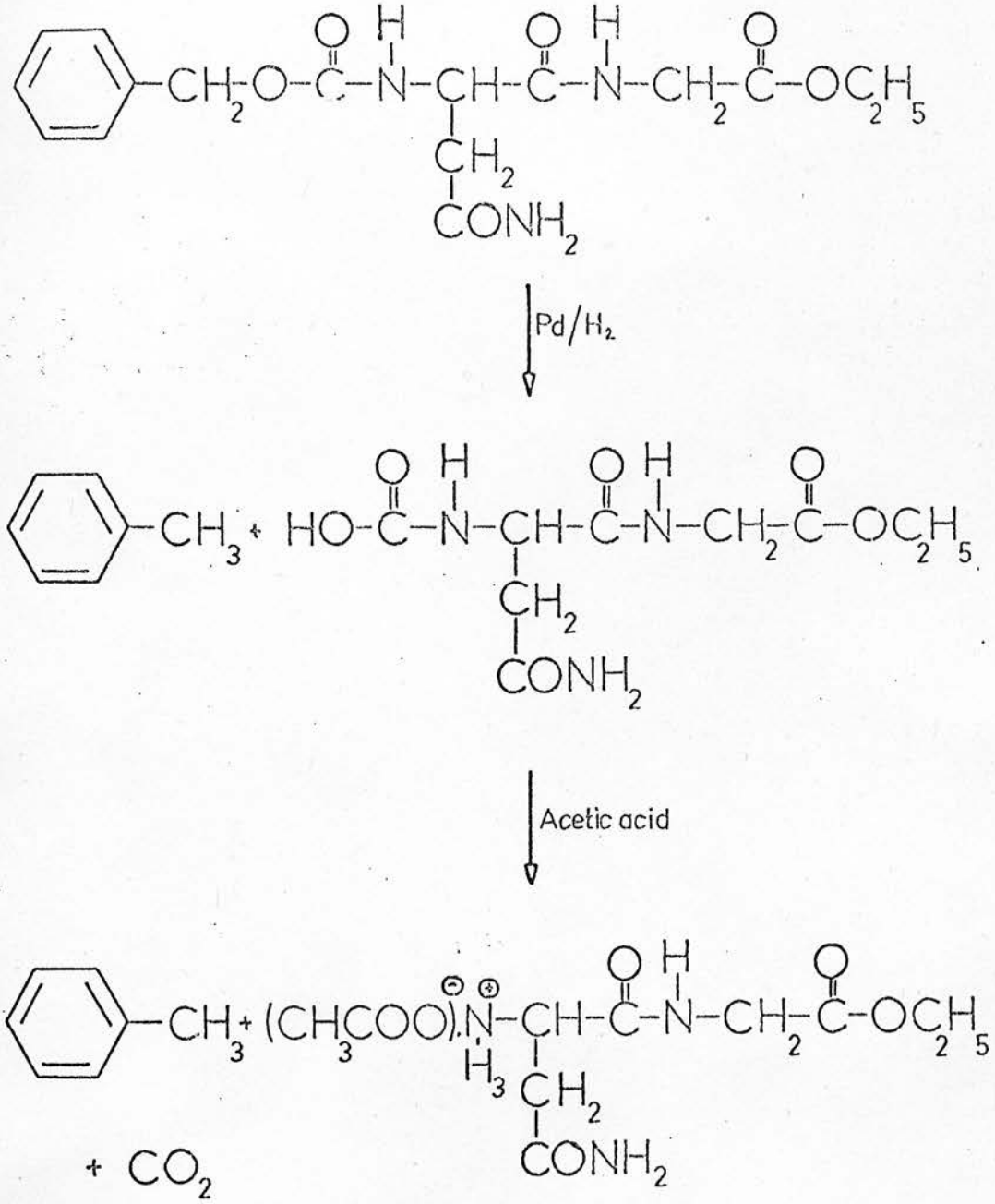
M.pt. - product decomposed at 230°C.

An IR spectrum was run on the white solid. A sample of the oil was investigated by T.L.C. and T.L.E., acid hydrolysis followed by amino acid analysis and LAP digestion. The results are shown in table 9.3 and confirm that the produce is in fact the required material. It was numbered S1.

This product can be used as the amino component for the synthesis of higher peptides. The compound Z-Gly-Asn-Gly-OEt was prepared. Z-Gly-O-Su was synthesized from Z-Gly (from BDH Biochemicals, the purity being confirmed by IR and NMR spectra) by a method analogous to that for the synthesis of Z-Asn-OSu. This was reacted with the above amino component as described for the preparation of Z-Asn-Gly-OEt to give a yellow solid. Recrystallization from methylene chloride and pet-ether gave a white solid which rapidly turned yellow on drying in air.

Catalytic hydrogenation of this product would give the unblocked tripeptide, Gly-Asn-Gly-OEt as its acetate salt.

Finally the preparation of Z-Gly-Asp-β-bz from Z-Gly-Asp-dibz was attempted. This was based on the method of Berger and Katchalski (1951) for the preparation of Z-Asp-βbz from Z-Asp-dibz[81]. Z-Gly-Asp-dibz (0.50 gms) was dissolved in a dioxan-water mixture (7.7 mls, ratio 5.2)



AcO-Asn-Gly-O-Et

Fig.9.2

and added to a solution comprising aqueous sodium hydroxide (0.5 mls, 2.5 M), water (2.7 mls) and dioxan (6.6 mls). The mixture was stirred and left at room temperature for 24 hrs. The pH was then adjusted to 5.5 by means of aqueous hydrochloric acid and the solvents were evaporated in vacuo. The residue was treated with aqueous sodium bicarbonate (10 mls, 1 M) and the mixture extracted with ether (15 mls) to remove unreacted dibenzyl ester. On acidification of the aqueous layer with hydrochloric acid (6 M) a fine white colloidal precipitate appeared. Solid sodium bicarbonate was added and the mixture redissolved. On reacidification a much heavier precipitate appeared and this was filtered, washed with a little water and dried. The solid was recrystallized from hot toluene.

m.pt. of start material - 83°C

m.pt. of product - 115°C

From the IR spectra it was seen that the expected product had not been produced (see table 9.2) but that the reaction sequence shown in fig. 9.3 had occurred.

The compound 5-carbobenzoxy-tetrahydro-1,4-diazepine3,7-dione was produced.

In the case of the preparation of Z-Asp-βbz in order to produce an analagous cyclic peptide the 7-membered ring would have to be replaced by a 4-membered one. This would be very much less stable than the above 7 membered ring.

The experiment was repeated using one molar equivalent of ammonia in methanol instead of the sodium hydroxide solution and using methanol as the substrate solvent. The reaction was again unsuccessful, a non-characterised, impure, white solid was produced. The use of

lithium hydroxide in aqueous acetone as base may be worth trying[35].

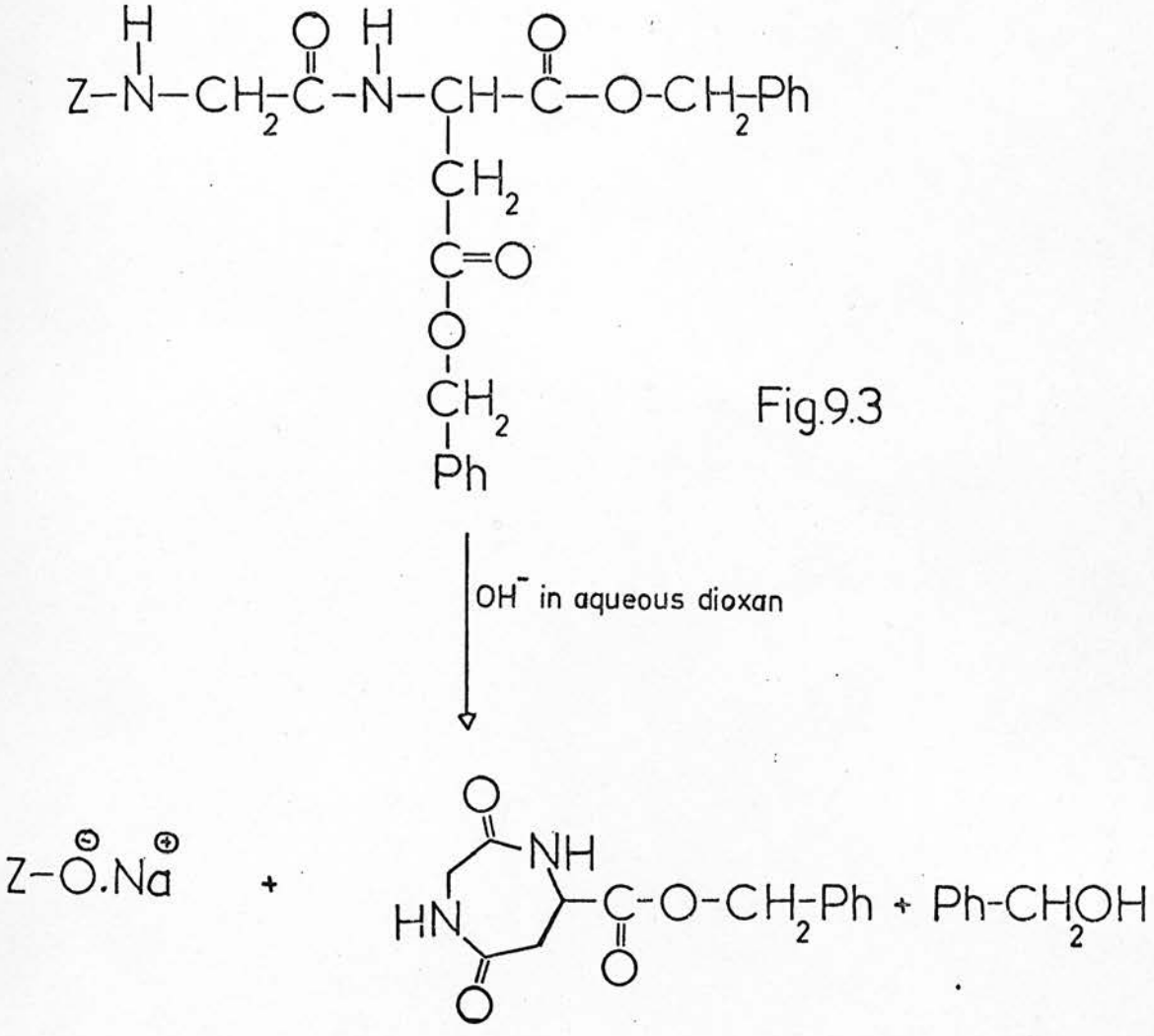


Fig.9.3

Infra-Red Absorption Spectra of Peptides and Proteins[75]

The infra-red absorption spectra of peptides and proteins are dominated by the characteristic bands of the peptide linkage. The position of the band is however modified by the specific environment of each group and especially by intermolecular hydrogen bonding and this can lead to band broadening. The effect of hydrogen bonding is to decrease the stretching frequencies and to increase the bending

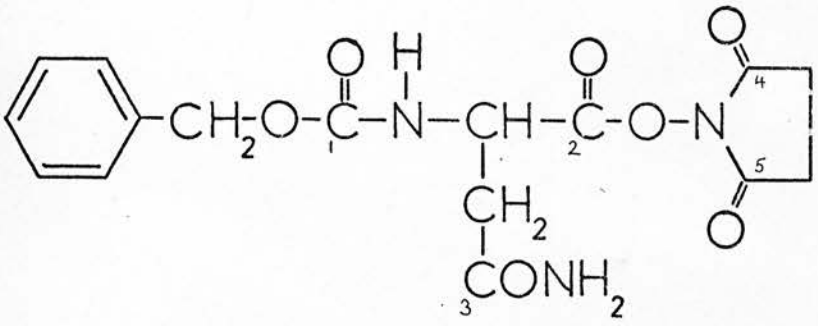
frequencies. Bonds due to specific side chain groups are discernible in the spectrum as well as the characteristic peptide absorption bonds.

Infra-red spectrometry was used to identify the products of synthesis and the results are given in tables 9.1 & 2. The spectra of the start materials were also run to help in assigning the spectral bands.

Table 9.1

Infra-Red Spectra of Synthesized Compounds

1. Benzyloxycarbonyl-asparaginyl-O-Succinimide

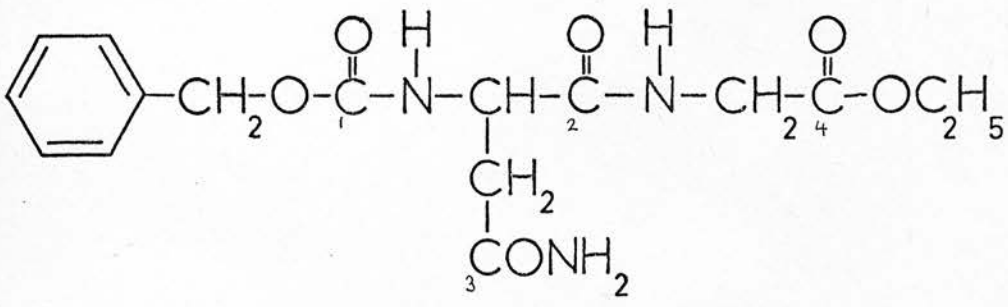


Frequency range considered: 1500 cm^{-1} to 4000 cm^{-1}

Frequency (cm^{-1})	Intensity	Interpretation
3450	W	N-H stretch - peptide bond
3350	W	$-NH_2$ Symmetrical stretch, side chain
3220	W	$-NH_2$ antisymmetric stretch, side chain
1810	W)	Succinimide carboxyl stretching
1795	W)	mode
1740	S	$_2C{=}O$ stretch
1730	S	$_3C{=}O$ stretch - side chain
1710	S	$_{4,5}C{=}O$ stretch - succinimide ring
1690	S	$_1C{=}O$ stretch

Table 9.1

2. Benzyloxycarbonyl-asparaginyl-glycyl ethyl ester



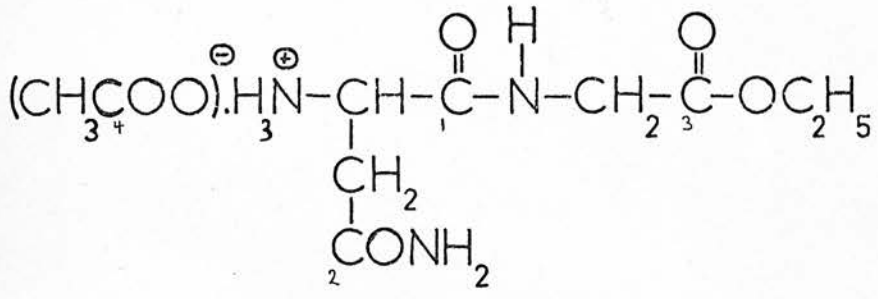
Frequency range considered: 1500 cm^{-1} to 4000 cm^{-1}

Frequency (cm^{-1})	Intensity	Interpretation
3450	W	N-H stretch
3400	W	N-H stretch
3350	M	$-NH_2$ symmetrical stretch - side chain
3280	W	$-NH_2$ antisymmetric stretch - side chain
1725	W	$_4C{=}O$ ester stretch
1715	W	$_3C{=}O$ stretch
1695	W	$_1C{=}O$ stretch Amide I
1670	S	$_2C{=}O$ stretch, Amide I
1655	M	H-bonded C=O stretch possibly
1650	M	
1630	W	
1565	W	Amide II involving $_1C$
1550	W	Amide II involving $_2C$

Note the absence of the succinimide carboxyl stretching frequencies.

Table 9.1

3. Acetate Salt of Asparaginyl-glycyl ethyl ester



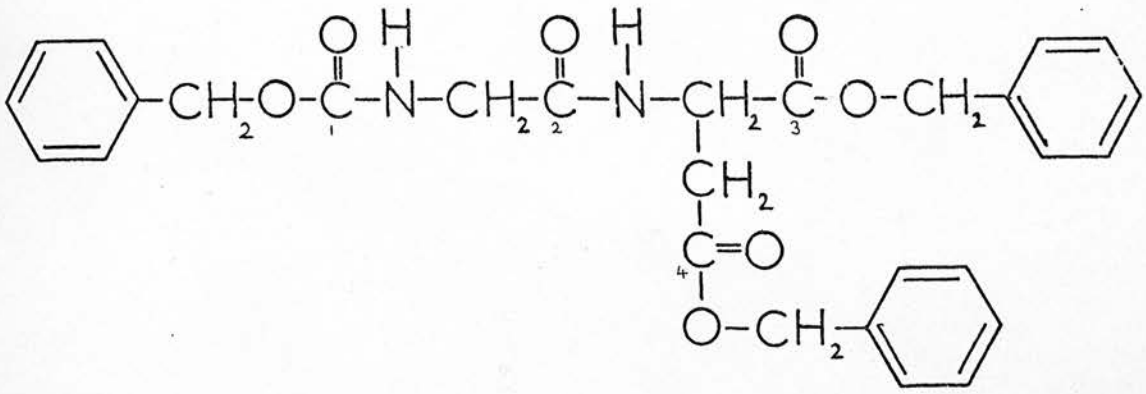
Frequency range considered: 1500 cm^{-1} to 4000 cm^{01}

Frequency (cm^{-1})	Intensity	Interpretation
3450	W	-N-H stretch
3370	W	$-NH_2$ symmetrical stretch, side chain
3230	M	$-NH_2$ antisymmetric stretch, side chain
1740	M	ester $_3C=0$ stretch
1710	M	$_2C=0$ stretch, side chain
1700	S	$_4C=0$ stretch
1692	S	$_1C=0$ stretch, Amide I
1680	S	H-bonded C=0 stretch possibly
1660	S	
1650	W	
1630	M	$\overset{+}{N}H_3$ deformation
1570	W	COO^- symmetrical stretching
1550	W	Amide II band involving $_1C$
1522	W	$\overset{+}{N}H_3$ deformation
(1420	W	COO^- antisymmetric stretching)

Table 9.2

Infra-Red Spectra of Compounds involved in the attempted Synthesis of Benzyloxycarbonyl-glycylaspartyl-β-benzyl ester

A. Benzyloxycarbonyl-glycylaspartyl-dibenzyl ester

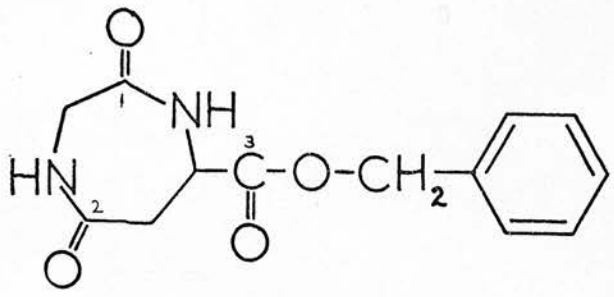


Frequency range considered: 1500 cm^{-1} to 4000 cm^{-1}

Frequency (cm^{-1})	Intensity	Interpretation
3300	S	N-H stretch
1742	S	ester $_3$C =O stretch
1730	S	ester $_4$C =O stretch
1692	S	$_1$C =O stretch
1650	S	$_2$C =O stretch
1550	S	Amide II involving $_2$C

Table 9.2

B. 5-Carbobenzoxy-tetrahydro-1,4-diazepine-3,7-dione



Frequency range considered -1500 cm^{-1} to 4000 cm^{-1}

Frequency (cm^{-1})	Intensity	Interpretation
3380	W	N-H stretch
3280	W	N-H stretch
1740	S	ester $_3$C=O stretch
1680	M	$_1$C=O stretch, Amide I
1648	S	$_2$C=O stretch, Amide II
1570	M	Amide II involving $_1$C
1550	M	Amide II involving $_2$C

Note:

Only 1 ester C=O stretch

Absence of acid C=O stretch

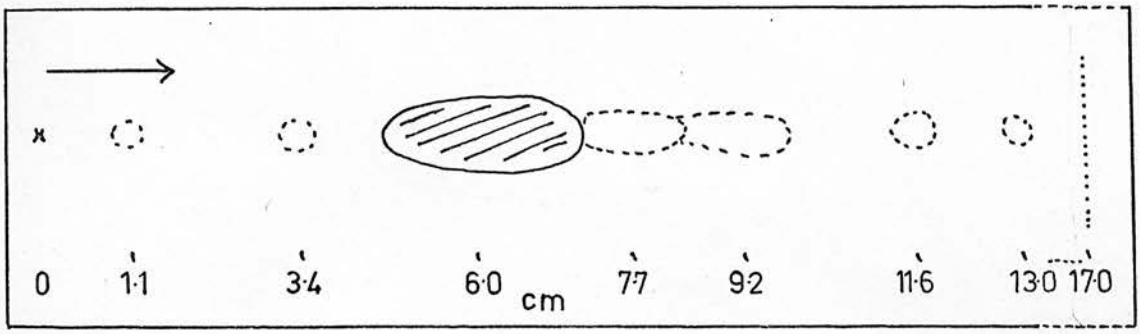
Absence of Z-group, on basis on loss of carbonyl groups.

Table 9.3

Initial Investigations on the Acetate Salt of Asparaginylglycyl ethyl ester

1. T.L.C.:- Stationary phase - MN-Cellulose 300G

Solvent - Butanol/Acetic Acid/Water 46/23/12



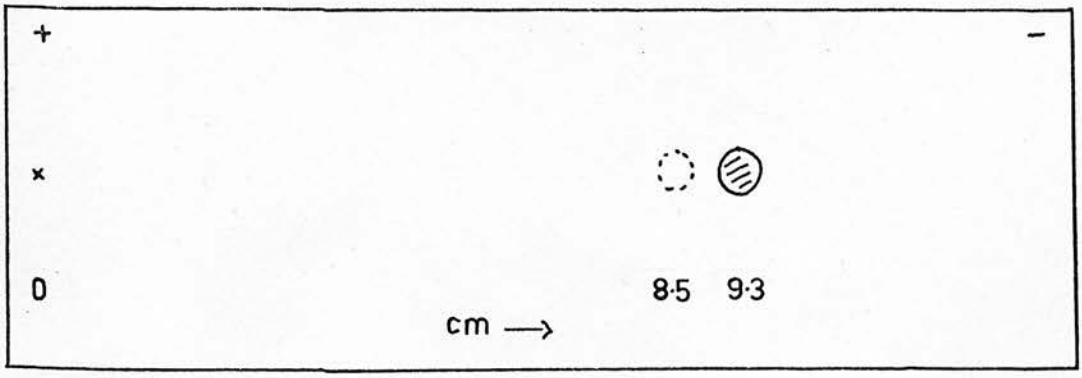
Main spot - very intense purple in colour.

Other spots - pale purple or brown.

2. T.L.E.:- Stationary Phase - MN-Cellulose 300G

Solvent - pH 2.2 pyridine formate buffer.

Electrophoresis details - 400 volts, 6 ma for 2½ hours (15 volts/cm)



both spots were purple in colour.

Table 9.3

3. Amino Acid Analysis :-

Amino Acids present	% ratio
Asp	41
Gly	59

4. LAP Analysis:-(See Section 10 for technique)

Digest time - 8 hours

Temperature - 37°C

Amino Acids present	% ratio
(A) Run	
Asp	2.5
Asn	56
Gly-OEt	41.5
(B) Control	
Asp	-
Asn	-
Gly	-

Section 10

Sequence Determination

When the amino acid analysis of a lysozyme or β-chain globin peptide matched that of one of the peptides being investigated in this study, its sequence was investigated further. The methods employed were those of LAP digestion, Dansyl-Edman sequencing, mass spectrometry and electrophoresis. These techniques also gave an indication of the degree of α → β aspartyl rearrangement that had taken place in the peptide.

(A) Leucine Aminopeptidase Digestion

LAP releases amino acids sequentially from the amino terminus of a peptide. It has been shown that there is a high rate of hydrolysis from amino acid residues with aliphatic or aromatic side chains, and lower, but still appreciable, rates from the other amino acids. The enzyme catalyses the hydrolysis of the peptide bonds of all amino acids with the exception of the imide bond of proline. The hydrolysis of αβ- or β-aspartyl residues is very slow and non-quantitative. By sampling the digest mixture after specific intervals of time a kinetic analysis of the liberation of amino acid residues was found and compared with the known peptide structure. The degree of aspartyl rearrangement was also gleaned from the LAP results.

The method used was based on that of Light[82]. Dry peptide (ca. 0.16 μmole) was dissolved in N-methyl morpholine acetate buffer (0.5 mls, 0.30 M in base, pH 8.6). Aqueous magnesium chloride solution (0.02 ml, 62.5 mM) was added and then the LAP solution introduced (0.025 mls, conc. 5 mg/ml in pH 8.6 buffer). Magnesium ions stabilize

the enzyme. The mixture was carefully shaken to dissolve fully the peptide and then incubated at 37°C in a capped vessel. A substrate control and an enzyme control were incubated at the same time. Samples of 0.1 ml were periodically removed, acidified with a little acetic acid to stop the reaction and then frozen until required. Sampling times varied from 20 minutes to 48 hours. The quantity of peptide initially used should be such that each sample contains approximately 0.03 μmoles of peptide so that peak areas can be measured fairly accurately.. The samples were analyzed using an amino acid analyzer, initially with a sodium ion buffer system but latterly with a lithium ion system; the details are given in table 10.1.

(B) Dansyl Edman Sequencing[83,84]

The phenylisothiocyanate (PITC) degradation of proteins is one of the most widely used methods for the analysis of amino acid sequencies of peptides. Basically it involves coupling the α-amino group of the peptide with PITC followed by acid catalyzed removal of the terminal residue. The newly exposed N-terminal group is coupled with dansyl chloride, then the peptide is hydrolyzed by acid and the dansyl amino acid residue identified by TLC or electrophoresis.

The reaction can conveniently be divided into six steps, the equations for which are shown in figs. 10.1-3.

(i) Coupling reaction with Phenylisothiocyanate

Samples containing approximately 5-10 nanomoles of peptide in solution were pipetted in appropriate Durham tubes. They were labelled with a diamond cutting pen at the base of each tube and then air and vacuum dried. Tube No.1 was set aside for dansylation. To each of the other tubes was added 50 μl of coupling mixtures (30 μl of PITC, 600 μl of pyridine, 400 μl of water; this was made fresh for each cycle using a stock solution of PITC in pyridine, 5% (v/v). They were covered

in parafilm, carefully shaken to dissolve all the peptide and incubated at 50°C for 45 minutes. The samples were next air dried and then vacuum dried at 70°C, the former procedure prevented flashing of sample.

(ii) Cleavage Step

To the dry samples was added anhydrous trifluoroacetic acid (50 µl) and the reaction was allowed to proceed at 70°C for 10 minutes. Excess reagent was removed by dry compressed air and the products were vacuum dried at 70°C.

(iii) Second Cycle

Tube No. 2 was set aside for dansylation and the cycle was repeated for the other tubes. The number of cycles performed was one less than the number of tubes initially taken.

(iv) Removal of Involatile bye-products

When all the cycles had been completed involatile bye-products (PTU, DPTU etc.) were removed. 40 µl of the aqueous phase of an ethyl acetate/water system (1:1 by volume) was added to all tubes except for number 1 and the bye-products removed by extraction with 3 x 200 µl of the ethyl acetate phase. The peptides were retained in the aqueous phase and were air dried and then vacuum dried.

(v) Dansylation

Each sample (including number 1) was dissolved in aqueous sodium bicarbonate solution (15 µl, 0.2 M) and then dried. This removed traces of ammonia which might have accumulated from the atmosphere or from reagents during the previous steps. The samples were redissolved in 15 µl of deionized, distilled water and the pH checked by means of indicator paper. If it was below pH 7.5-8 more sodium bicarbonate was added since dansylation is not satisfactory below this range. Dansyl

chloride solution (15 µl, 3 mg/ml in analytical grade acetone) was added and the solutions were carefully mixed. The tubes were covered in parafilm and left for 15 minutes at 50°C. They were then air and vacuum dried at 70°C.

Redistilled, constant-boiling HCl (100 µl) was added to each tube and these were then sealed in a fine flame. Hydrolysis was allowed to proceed overnight at 105°C.

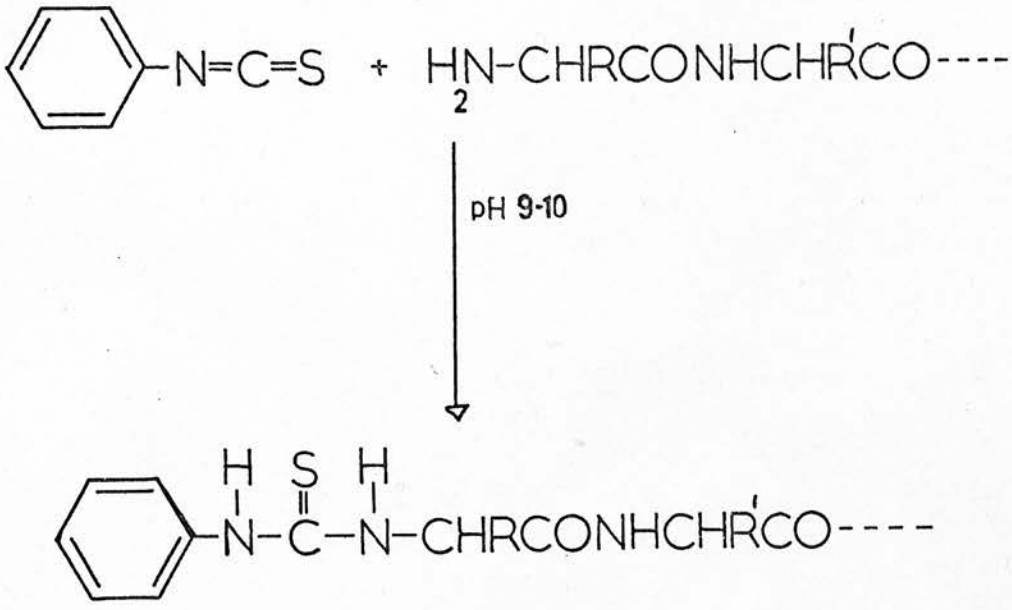
(vi) Identification of end groups

The tubes were cooled before they were opened and the HCl removed under vacuum. The dansyl-amino acids were extracted with the organic phase of the ethyl acetate/water system and transferred into duplicate labelled tubes. Two extracts were taken (2 x 100 µl) and the aqueous material was set aside. The ethyl acetate was removed by evaporation at 85°C and the dansylated amino acids dissolved in 50% aqueous pyridine (15-20 µl). The residues were identified by thin layer chromatography using polyamide TLC plates[85] and a benzene/acetic acid (9:1) solvent. The spots were observed under UV radiation.

DNS-Arg, in company with DNS-$CySO_3H$, α-DNS-His and α-DNS-Lys, is only found in trace amounts in the ethyl acetate phase, and in this case the sample was not extracted but dissolved in 50% aqueous pyridine and spotted on TLC plates complete with salts.

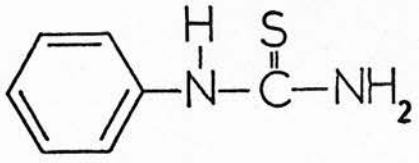
The test dansyl amino acids were compared both with standards and also literature positions. The one solvent system used in this work will separate the amino acids contained in the peptides being investigated. A 4-solvent procedure is necessary to separate all the products of the naturally occurring amino acids.

Coupling reaction

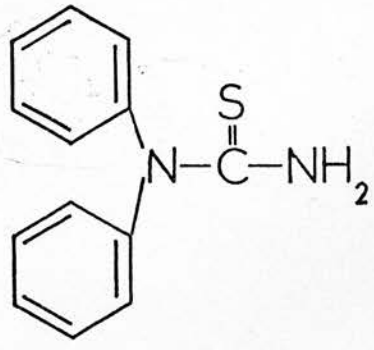


N-phenylthiocarbamoyl peptide

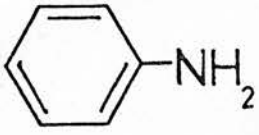
By products include:



phenylthiourea (PTU)



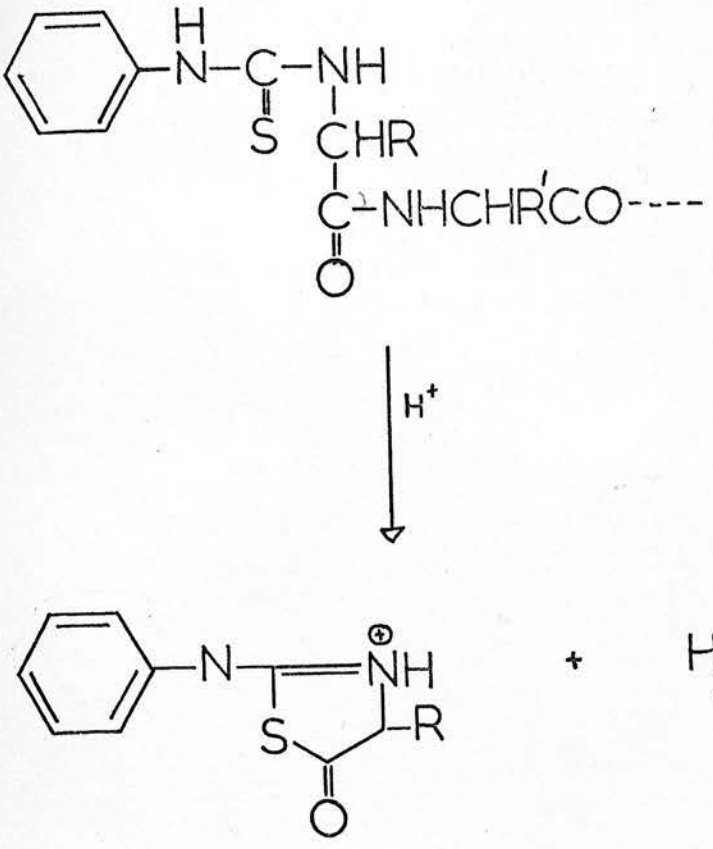
diphenylthiourea (DPTU)



aniline

Fig.10.1

Cleavage step



2-anilino-5-thiazolinone derivative

newly exposed N-terminal amino acid

Fig.10.2

Dansylation

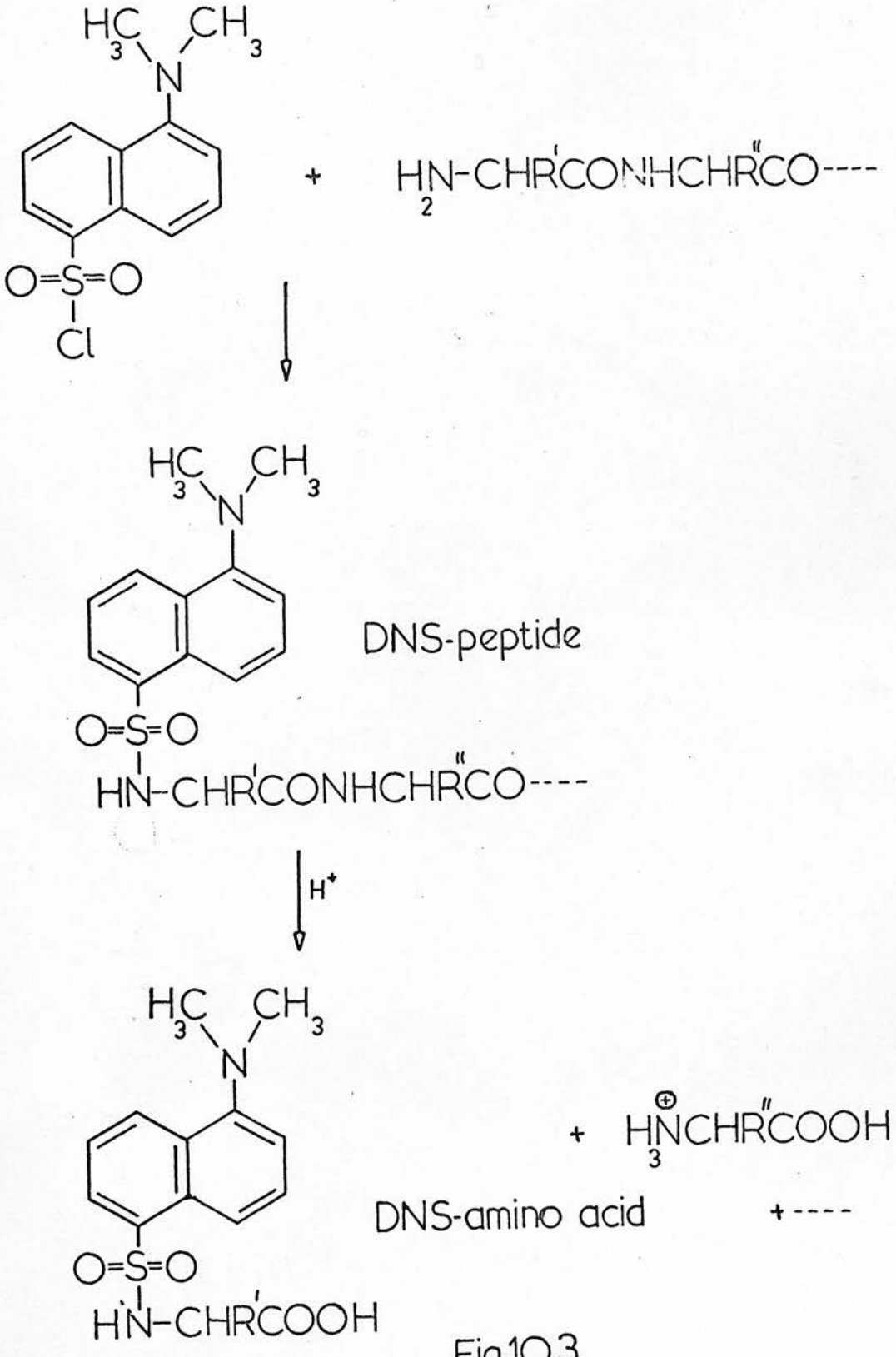


Fig.10.3

(C) Mass Spectrometry[86]

Peptides are not sufficiently volatile to be studied directly by mass spectrometry, but this technique can be used if the amino groups and carboxyl groups are blocked by acetylation and permethylation. In this laboratory, a precautionary degreasing of the sample was performed before undertaking the volatilization procedure. The equations are shown in figure 10.4.

The peptide (ca 0.15 μmole but not less than 0.10 μmole) was dissolved in 50% acetic acid (1 ml) and the grease was extracted with 3 x 1 ml lots of redistilled pet-ether. The peptide was dried in vacuo at room temperature and then dissolved in 1 ml of acetic anhydride in methanol (1 : 4 by volume). The mixture was carefully shaken to dissolve the peptide and left at room temperature for 3 hours. The acetylated peptide was finally air dried.

The N-terminus, along with the side chain amine groups of lysine and β-ae-cysteine, if present in the peptide, are acetylated.

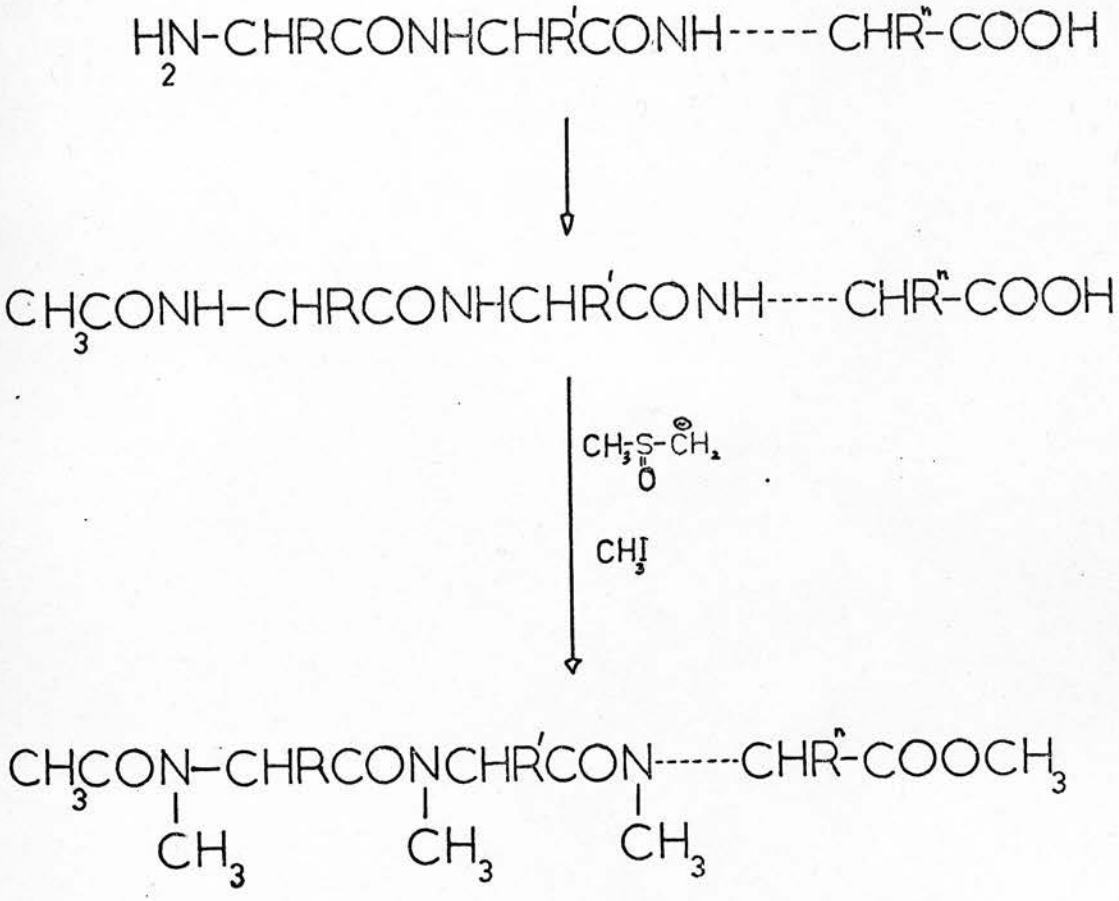
The acetylated peptide was dissolved in dry, redistilled dimethyl sulphoxide (0.1 ml), and put aside. Sodium hydride (20 mgs, dry and kept under dry nitrogen) was added to 1.5 mls of DMSO and the mixture placed in an oven at 70°C. When it had turned a golden-yellow in colour (after about 30 minutes), it was centrifuged and 0.5 mls of the clear supernatent, containing methyl sulphinyl carbanion was withdrawn and added to the peptide solution. Methyl iodide (0.5 mls, redistilled and kept over copper) was added and the mixture was shaken gently at room temperature for 1 hour. It is not advisible to leave the reaction for longer than 1 hour since C-methylation of, for instance, glycine may occur. The reaction was terminated by adding deionized

water (1 ml) to the mixture and the peptide was extracted with redistilled chloroform (0.5 mls). The chloroform layer was washed twice with water and then air dried. The acetylated, permethylated peptide was finally dried in a vacuum desiccator over phosphorus pentoxide and then analyzed with the mass spectrometer. Side chain hydroxyl, amide and carboxylic acid groups are also methylated.

(D) Electrophoresis

Electrophoresis at pH 3.5 may distinguish between α-aspartyl and β-aspartyl peptides. Both paper and thin layer electrophoretograms were run, and the conditions are given in table 10.2. In both cases two samples of the same natural product peptide were run side by side, a small analytical sample and a larger preparative sample. The former was sprayed with ninhydrin and the positions of the spots noted. The latter was cut into strips corresponding to these positions, in the case of the paper electrophoresis. The peptide contained in these strips was eluted into vials with acetic acid (0.1 M). The solutions were air-dried and the material digested with LAP for 24 hours. In the case of the thin layer plates, the cellulose in the preparation run corresponding to the position of peptide in the analytical run was scraped off the plate and washed with acetic acid (0.1 M). The washings were treated as above.

The LAP digests were analyzed on the lithium column and the results are given in tables 10.3-7.



acetylated, permethylated peptide

Fig. 10.4

Table 10.1

LAP Analysis Details

	Column 1	Column 2
Resin	Technicon "A" 25 μ	Technicon "A" 18 μ
Column Dimensions	56 x 0.636 cms	64 x 0.636 cms
Jacket temperature	39.5℃	39.5℃
Buffer ion and concentration	Sodium, 0.2 N	Lithium, 0.3 N
Starting buffer pH	3.28 ± 0.01	2.80 ± 0.01
Second buffer pH	4.25 ± 0.02	4.15 ± 0.01
Buffer Flow Rate	30 mls/hr.	30 mls/hr.
Back pressure	110 p.s.i.	180 p.s.i.
Buffer timer change	60 mins.	3 hours
Regenerant	0.2 N NaOH	0.3 N LiOH
Wash buffer, pH	3.28 + 10% MeOH	2.80 + 10% MeOH or 10% dioxan

Basic Column Analysis - details as described in table 1.1

Table 10.2

Electrophoresis Conditions

(a) Paper Electrophoresis

(i) Whatman No. 1 Chromatography paper with No. 3 MM connection pieces.

(ii) Pyridine Acetate buffer, pH 3.5, 0.124 M in base.

(iii) Voltage - 3 kv (35 Volts/cm)

(iv) Current - 30 ma.

(v) Time - 3 hours.

(b) Thin Layer Electrophoresis

(i) Stationary Phase - MN-Cellulose.

(ii) Pyridine Acetate buffer, pH 3.5, 0.124 in base.

(iii) Voltage - 600 V (22 volts/cm).

(iv) Current - 18 ma.

(v) Time - 3 hours.

Both systems were water cooled.

Section 11

α→β-Aspartyl Rearrangement

It was decided to investigate the α→β-aspartyl rearrangement of the synthetic and natural product peptides by treatment of these peptides with organic acids, bases and buffers under varying conditions of time, temperature, pH, buffer base concentration and base. The precise details are given in tables 11.2-10.

If the synthetic peptide, asparaginyl-glycyl ethyl ester, is considered first, this peptide, in aqueous solution, was kept frozen when not in use. To one ml of the thawed solution was added an internal standard of DL-Valine to a concentration of 11.9 mg per 100 mls which corresponds to 1.106 μmoles/μl. By comparison with norleucine (0.50 mls = 0.100 μmoles) a value for the concentration of 1.103 μmoles/μl was obtained. The basic procedure was the same for all the runs and the example of variable buffer base concentration for constant base, pH, temperature and time will be given.

Ten samples (10 μl) of the peptide solution were pipetted from the stock solution and air-dried in small vials. To pairs of samples were added the buffers to be tested for rearrangement activity (0.2 mls); in this example they were pyridine acetate buffers, pH 5.0 and with base molarity varying from 0.1 M to 5 M. They were sealed and incubated at 50°C for 42 hours. After this time the samples were air dried and dissolved in N-methyl morpholine acetate buffer (0.2 mls, 0.3 M pH 8.6). Magnesium chloride solution (10 μl, 62.5 mM) was added and then LAP solution (10 μl) added to one of each pair of samples. All ten samples were incubated at 37°C for 2½ hours, this time being found adequate for complete cleavage of the asparaginyl-glycine bond by prior experiment.

The samples were then kept in a frozen state until required. Immediately prior to use, the samples were thawed and acidified with acetic acid and then loaded onto a column of Amberlite CG120 to separate the constituent amino acids. Table 11.1 gives full details. Three samples could be analysed before regeneration of the column was necessary. Norleucine was not present in the mixture but the condition of the ninhydrin was monitored each day from the trace of an amino acid analysis. The results were worked out for constant values for both norleucine and valine. A time zero run was performed using 25 µl of sample for both run and control and LAP digestion. To check on the amount of rearrangement induced merely by the basic conditions of the LAP digestion, an acid hydrolysis of 25 µl of sample was performed. An untreated sample was also separated on the CG120 column and a sample of peptide which had been incubated with 1M pyridine for 6 hours at 50°C was separated on the Technicon "A" 18µ column using the conditions of Dorer et al (1967) given in table 11.1.

The kinetic runs using natural product material were performed in a similar manner to the synthetic peptide runs. Only one internal standard was used (norleucine). The experiments were conducted at room temperature and a LAP incubation time of 4 or 6 hours was used. The results were analyzed on the long Technicon "A" 18 µ lithium column and the results are given in tables 11.11 & 12.

The use of polarimetry as a monitoring technique for the rearrangement was tried but found to be unsuitable. A Perkin-Elmer 141 instrument was used with a mercury lamp and a wavelength at $436cm^{-1}$. However the concentration of material used (ca 0.18 µ moles per ml) was too small for the instrument.

The results will be discussed in the next section.

Table 11.1

Synthetic Peptide Base Runs Analysis System

	Column 1	Column 2
Resin	Amberlite CG120	Technicon "A" 18 μ
Column Dimensions	15.0 x 0.45 cms	64 x 0.636 cms
Jacket temperature	55°C	60°C
Buffer ion and concentration	Sodium, 0.2 N	Sodium, 0.5 N
Buffer pH	3.25 ± 0.01	1.82 ± 0.01
Flow rate	30 mls/hr	30 mls/hr
Back pressure	50 p.s.i.	120 p.s.i.
Regenerant	0.2 N NaOH	0.5 N NaOH
Wash buffer pH	3.25	1.82

RESULTS and DISCUSSION

Nomenclature

The following nomenclature will be used in describing the peptides encountered in this work:

Ly - lysozyme peptide

β - β-chain globin peptide

T - tryptic peptide

C - Chymotryptically cleaved tryptic peptide.

Number immediately after Ly or β refers to the digest batch number.

Number immediately after T refers to the tryptic peptide number starting from the N-terminus.

Subscript number following C refers to the first, second, third etc., chymotryptic peptide in the tryptic peptide from the N-terminus.

e.g. Ly4C,T10 - lysozyme peptide, batch 4, the first chymotryptic peptide in tryptic peptide number 10.

The numbered tryptic peptides for both lysozyme and β-chain bovine globin were listed in tables 7.2 & 3.

S1 - Synthetic peptide, (CH_3COO^-). $^+$Asn-Gly-OEt, batch 1.

In most cases, and in all cases where ambiguities may occur, the sequence of the peptide will also be given.

Peptide Separations

The separation of the tryptic digest of β-ae-lysozyme 2 on Zeo-Karb 225 8% cation exchanger is shown in fig. 8.1 and that for β-ae-bovine globin β-chain 1 on the same resin is shown in fig. 8.2. Fractions from both these, and those involving lysozyme batches 1 and 3 and β-chain batch 2 were refractionated on Amberlite 200 cation exchange resin and a cross section of the runs are illustrated in figs. 8.3-16. The sequences of ae-lysozyme and ae-bovine globin β-chain are shown in figs. 8.17-19.

Zeo-Karb 225 8% is a conventional cation exchange resin and separation is achieved both on the basis of size and acidity; large peptides are eluted before smaller ones and acid peptides are eluted before basic ones. The tryptic digest separations of the lysozyme preparations were observed to be similar but not identical. This was probably due to the fact that exclusively tryptic digestion was not taking place and some Q-tryptic and chymotryptic activity was observed in the enzyme preparations used.

The same phenomena was observed in the case of the β-chain separations. However, a given peptide should emerge from the columns at a given value of buffer base molarity and buffer pH. Using this fact, information derived from one run can be combined with that obtained from another, as in tables 8.4 and 5.

Certain irregularities to the size/acidity expected peptide order off the column were observed. For instance, the peptide T6-His-Glu-Leu-Asp-Asn-Tyr - is a medium sized neutral peptide and yet it was eluted from the Zeo-Karb column with 2,3 and 5-membered basic peptides and after several 2-7-membered basic peptides. This is possibly due to the presence of histidine and tyrosine residues slowing the rate of progress of the peptide down the column. Phenylalanine containing peptides were also observed to be "slow" but this did not seem to apply to the tryptophan containing ones which was unexpected. Some basic peptides, for instance T17, Ala-Lys, T24, Gly-Ae-Cys, were observed to emerge from the column sooner than expected. This has been observed before; Schroeder[57] noted that in the separation of this substrate on Dowex 50 x 2, lysine, (T8) was observed to emerge in the peptide band corresponding to the first large peak. Other peptides in this peak included T14, a 19-residue acidic peptide. Jones[87] found that lysine

was separated from a mixture of human β-chain globin tryptic peptides on a column of Spinco type 15A (x8) resin before T11, a 7-residue neutral peptide. Perhaps with free amino acids and very small peptides the molecular sieving properties of the resin are reduced since their exit from the 'pores' is not hindered. Dowex 50 x 2 and Spinco type 15A x 8 are similar types of resin to Zeo-Karb 225; the degree of cross-linking is reduced in the former case but identical in the latter.

In the case of the Amberlite 200 resin there did not appear to be any separation on the basis of size as shown in figures 8.3 - 15 for example. This was to be expected since this macroreticular resin contains very large pores which can accommodate large peptides. Separation purely on the basis of acidity was not clearly observed and the amino acid composition of the peptide seemed to be important. Histidine, tyrosine and phenylala ine containing peptides, for instance, again tended to be late off the column. In separation 2JK (fig. 8.11) the emergence of C_3T10, a 5-membered basic peptide of sequence Gln-Ile-Asn-Ser-Arg, before the neutral dipeptide Gly-Tyr can possibly be explained by the slight acidity of the two amides, glutamine and asparagine. Any acidity derived from hydroxyl groups would be cancelled by the presence of serine in one peptide and tyrosine in the other. The delaying effect of tyrosine will also be connected with the apparent reversal of order.

With both resins a peak was observed at, or just after, the void volume. This material was often a mixture of peptides that were difficult to characterize. Sometimes the mixture seemed to comprise peptides that were separated in a purer state later in the chromatogram. However in the case of run Ly2H (fig.8.10) the later peaks apparently

corresponded to portions of the peptide responsible for the initial peak. The initial peak corresponded to a 12-residue neutral peptide whilst the second was a 5-residue acidic one (albeit containing two phenylalanine residues) and the third a basic heptapeptide. There is then a possibility that the original 12-membered peptide was separated on the Zeo-Karb column and cleavage after the second phenylalanine residue occurred whilst preparing the peptide for refractionation. A peak immediately after the void volume had been eluted in the Zeo-Karb separation of the tryptic digest could correspond to urea if this material had not been fully removed by dialysis after the aminoethylation step.

Because of the number of peptides present in the digest - attributable to pseudotryptic as well as tryptic digestion - some peptide mixtures were very difficult to characterise. In cases where there was obvious overlap from neighbouring peaks the principal constituent only was mentioned in the tables. In a few cases reliable interpretation could not be made. Frequent amino acid contaminents in the lysozyme peptides were aspartic acid, serine and glycine, and in the β-chain peptides, aspartic acid, glutamic acid and glycine. The physical properties of the two columns, especially the Amberlite 200, were good and both gave trouble-free service for over two years.

The separation afforded by the anion exchanger, "Deacidite" FFIP of the acidic peptide mixture LylAa was poor (fig. 8.16). The anion exchanger would be better employed in separating mixtures of basic peptides rather than acidic ones. Refractionation of the Amberlite peptides on the Zeo-Karb 225 8% column using a modified gradient would probably be more satisfactory for the purification of acidic peptide mixtures.

The kinetics and selectivity of peptide exchange are rather complex, but it can be clearly seen that the successive use of the conventional and macroreticular resins has afforded good separation of peptide mixtures.

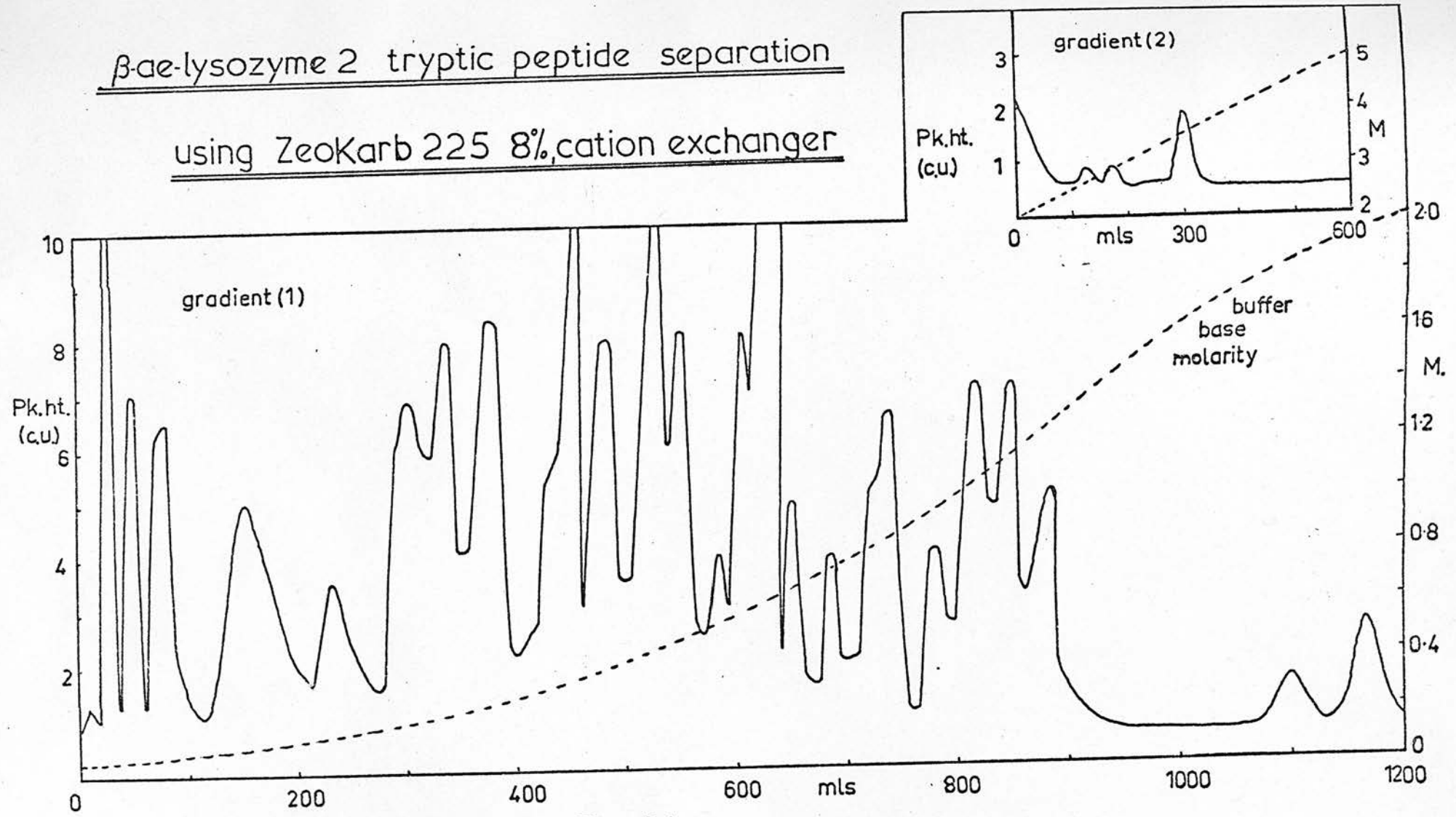

β-ae-lysozyme 2 tryptic peptide separation
using ZeoKarb 225 8%, cation exchanger
gradient (1)
gradient (2)
Pk.ht. (c.u.)
buffer base molarity
M.
mls
0
200
400
600
800
1000
1200
2
4
6
8
10
1
3
5
16
1·2
0·8
0·4
300
140.

Fig. 8.1

Bovine β-chain tryptic peptide separation using ZeoKarb 225 8% cation exchanger

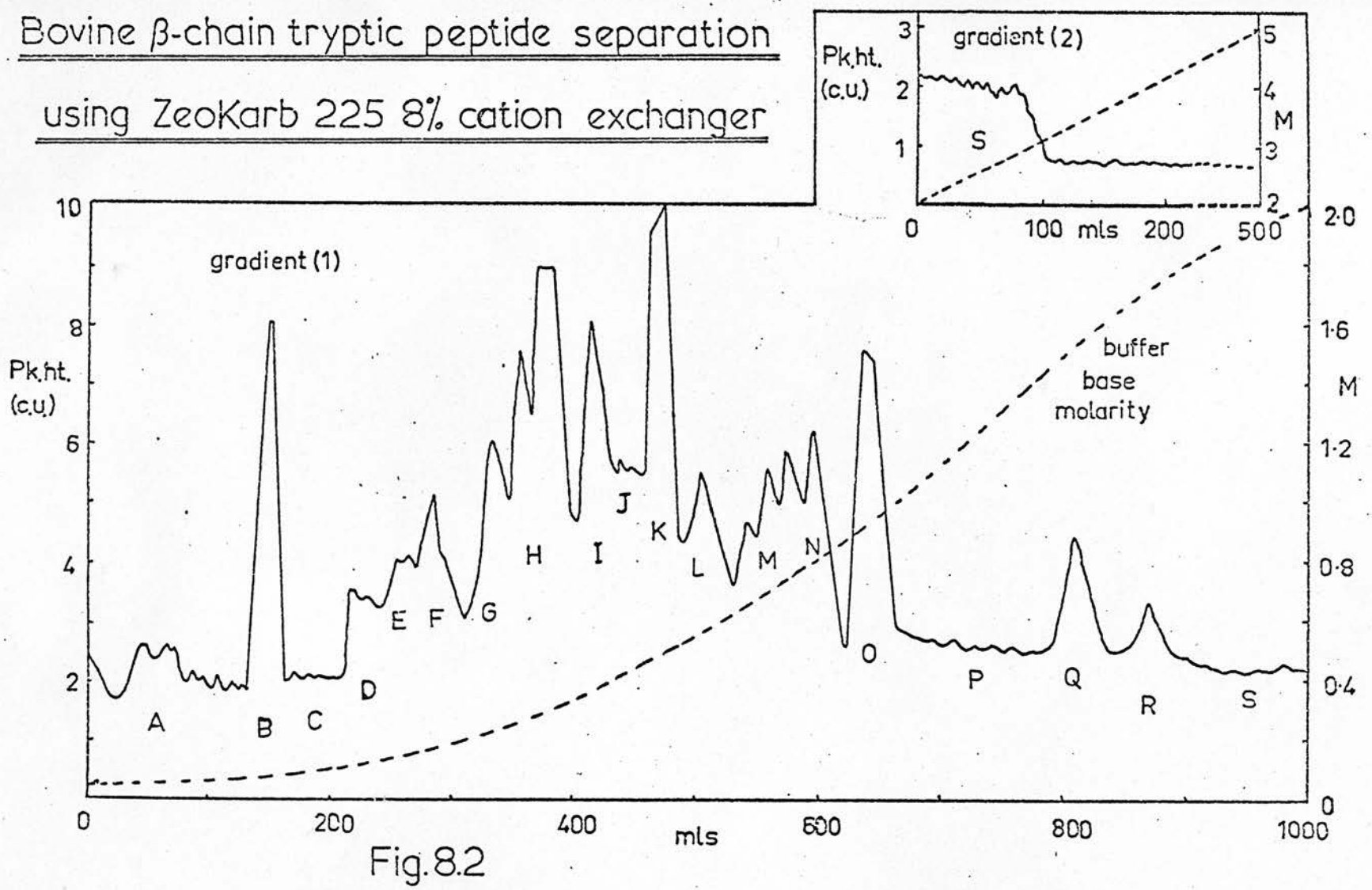
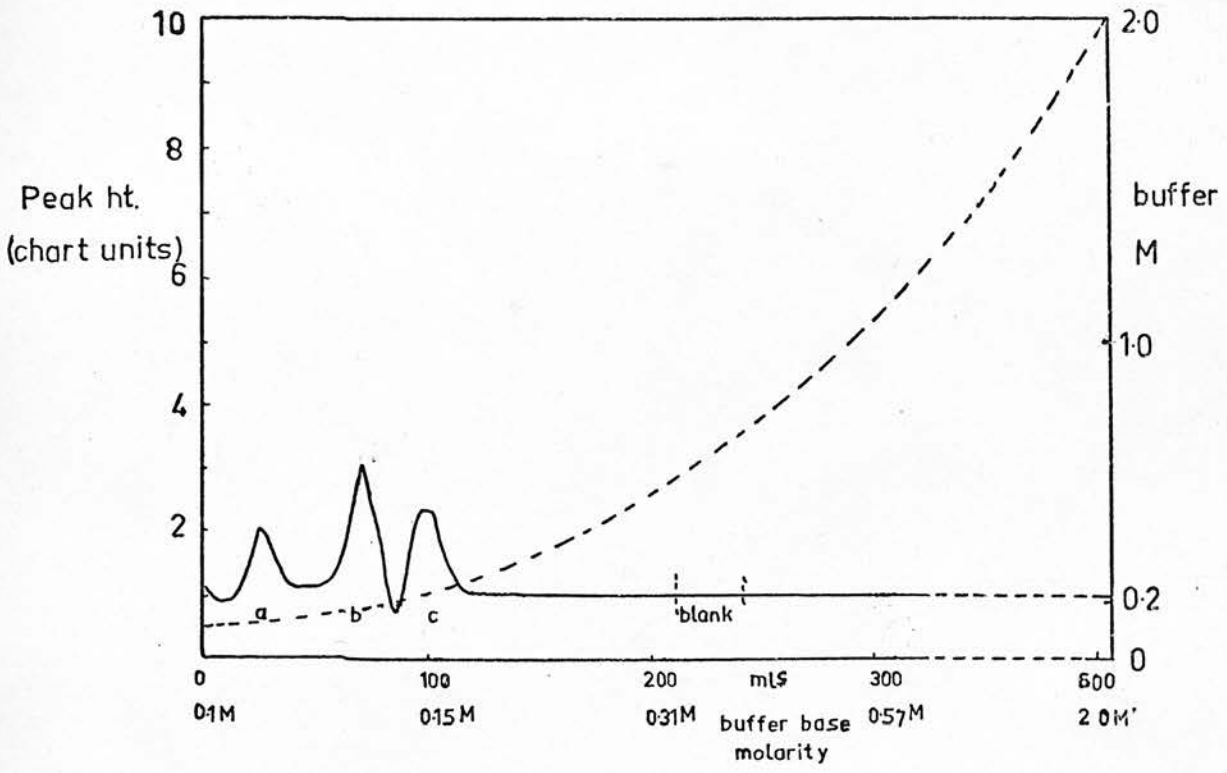


Fig. 8.2

Fig 8.3

Amberlite 200 Peptide Separation : Lysozymelc peptides : Concave gradient



a - Void volume material

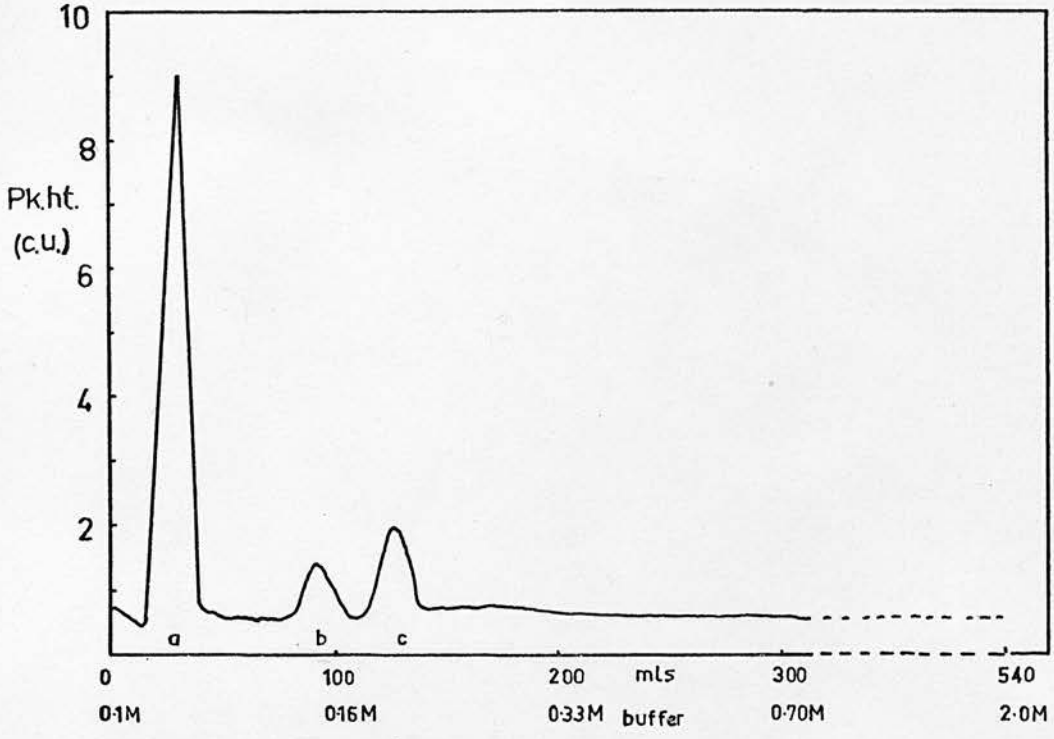
b - non-characterized peptides

c - C_2T16 - Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Ae-cys

blank - traces of Asp, Ser and Gly present

Fig. 8.4

Amberlite 200 peptide separation : Lysozyme tE peptides : Concave gradient



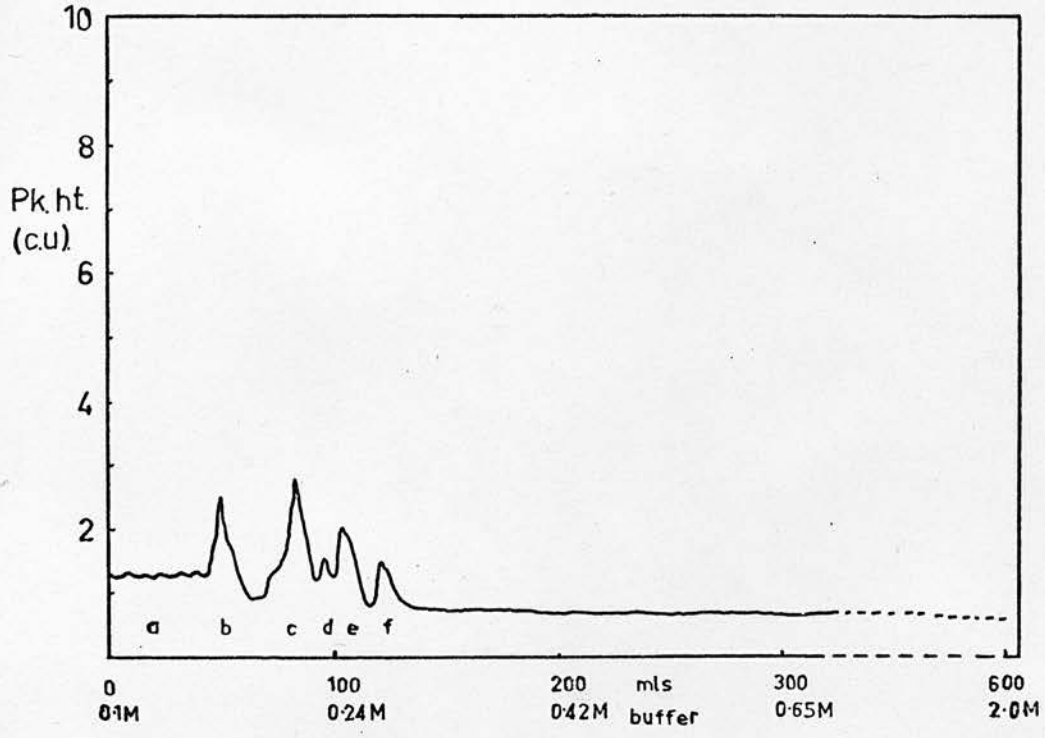
a - C_1T23 plus C_1T16 ie Ser-Ala-Leu-Leu

b - Miscellaneous non-characterized peptides

c - C_1T23 ie Gly-Thr-Asp-Val-Gln-Ala-Trp

Fig. 8.5

Amberlite 200 peptide separation : Lysozyme 1H peptides : Concave gradient



a - Void Volume material

b - C_1T7 - ie Gly-Tyr

c - T15 ie Asn-Ile-Pro-Ae-cys

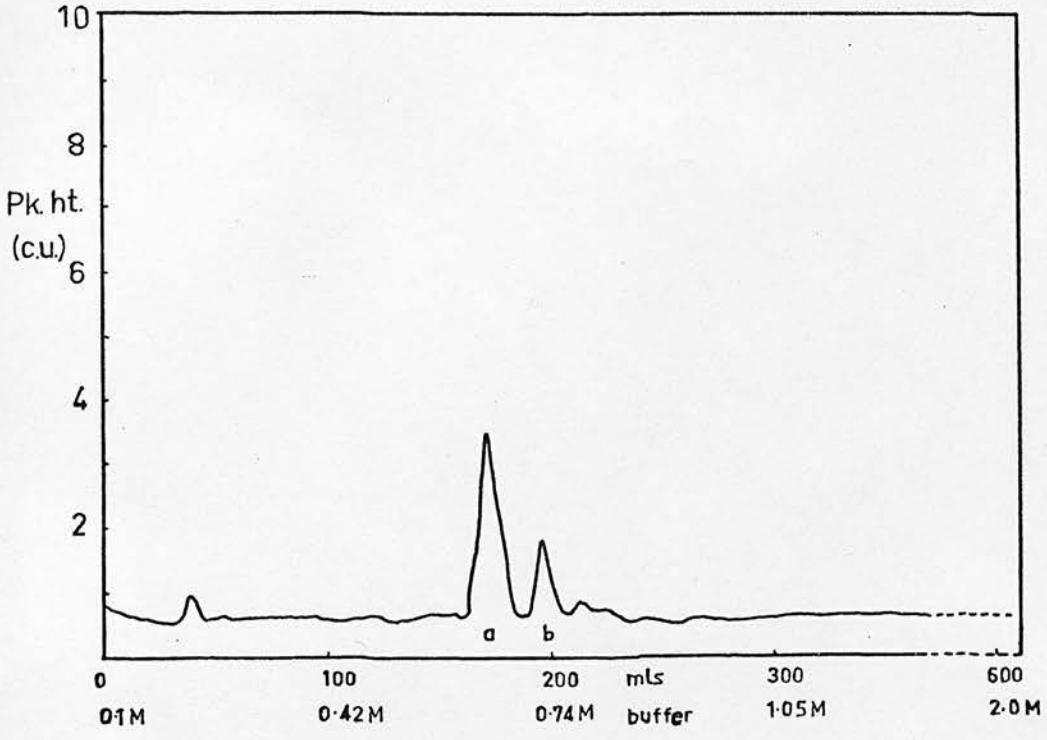
d - *

e - C_3T11,12 ie Ae-Cys-Asn-Asp-Gly-Arg (impure)

f - C_3T11,12 ie Ae-Cys-Asn-Asp-Gly-Arg

Fig. 8.6

Amberlite 200 peptide separation : LysozymelJ peptides : linear gradient

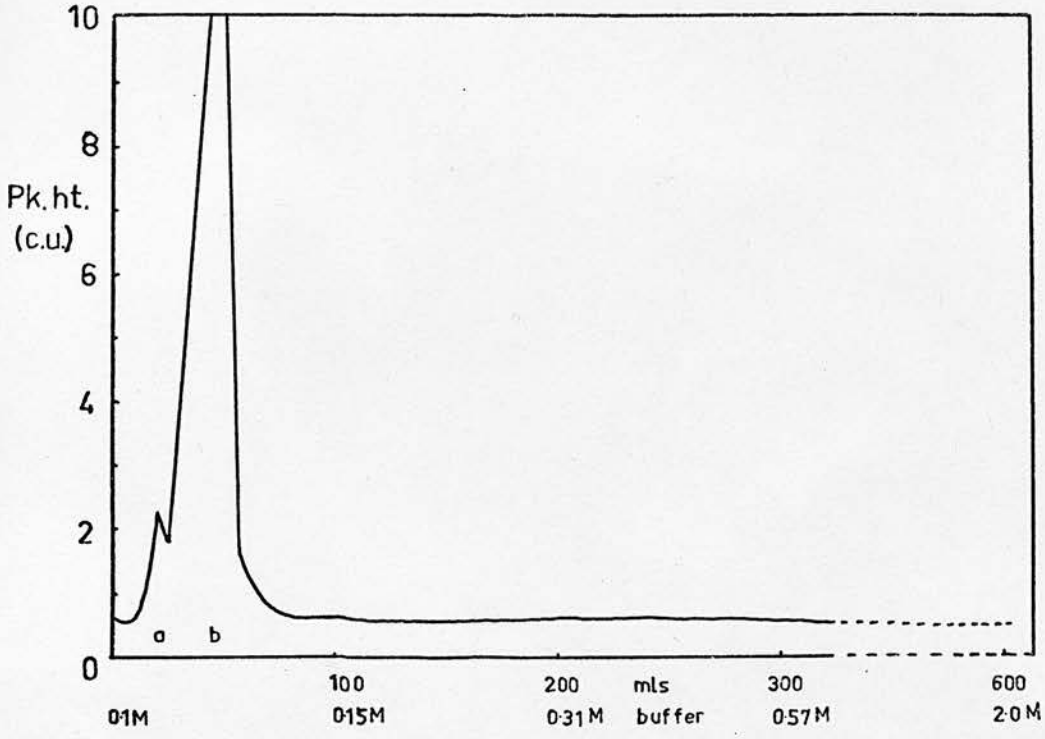


a - T13 ie Thr-Pro-Gly-Ser-Arg

b - T14 ie Asn-Leu-Ae-Cys

Fig 8.7

Amberlite 200 peptide separation : lysozyme 2D : Concave gradient

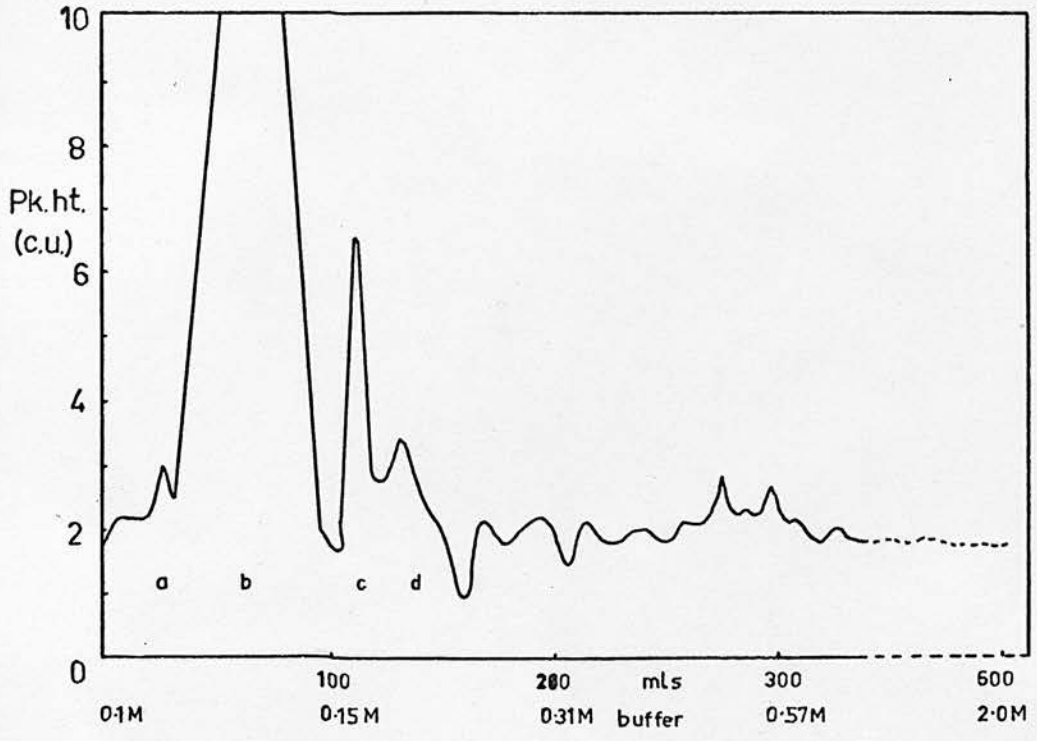


a - Void volume material

b - C_1T10 ie Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

Fig. 8.8

Amberlite 200 peptide separation : lysozyme 2E peptides : concave gradient



a - Void volume material

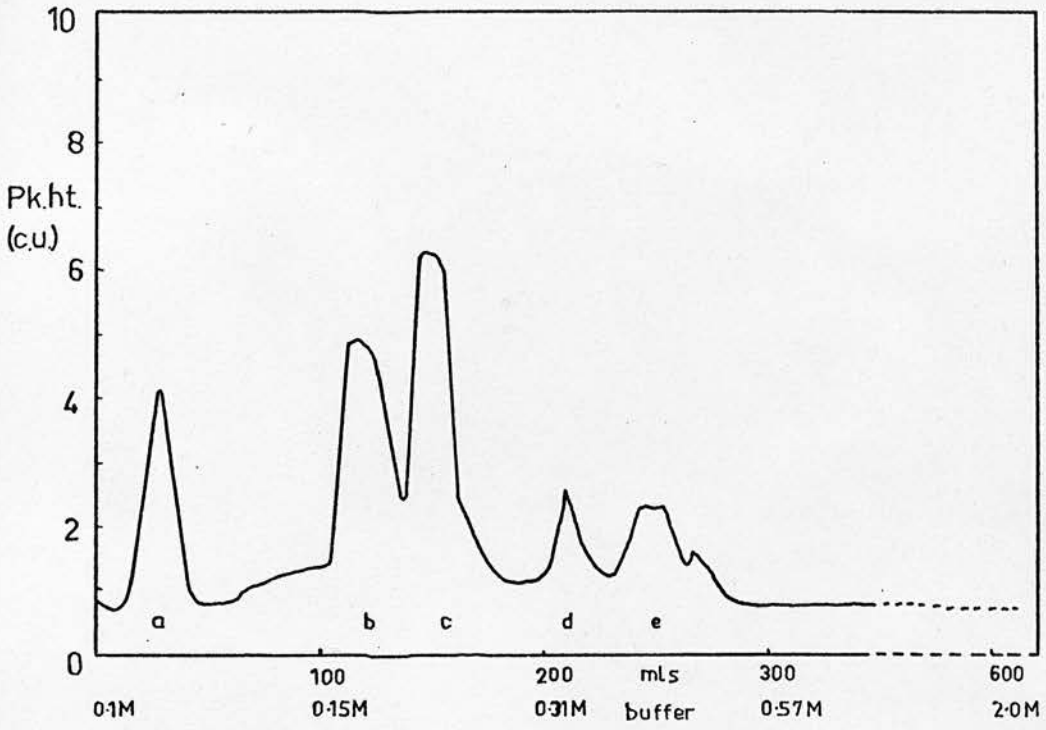
b - C_1T10 ie Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

c - C_1T19 ie Ile-Vel-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp

d - miscellaneous peptide material

Fig. 8.9

Amberlite 200 peptide separation : lysozyme 2G peptides : concave gradient



a - Void volume mixture.

b - C_1T16 ie Ser-Ala-Leu-Leu + C_2T9 Gln-Ser-Asn-Phe

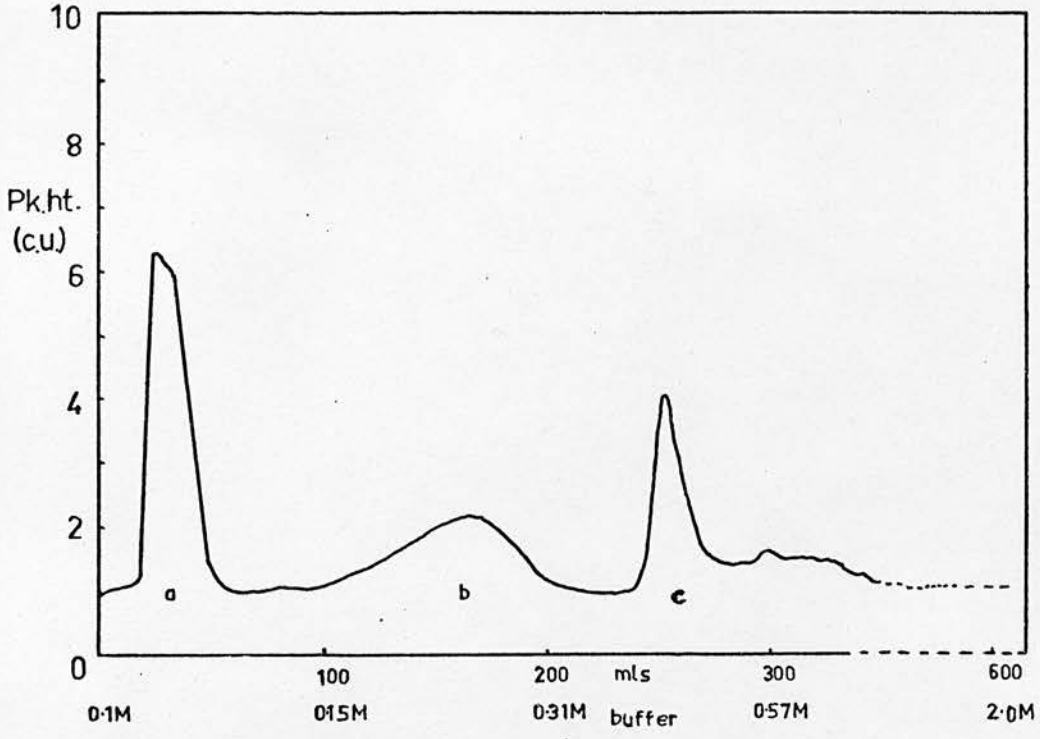
c - C_1T23 ie Gly-Thr-Asp-Val-Gln-Ala-Trp

d - C_1T9 ie Phe-Glu-Ser-Asn-Phe

e - C_3T7 ie Gly-Asn-Trp(?) + C_3T9 ie Asn-Thr-Gln-Ala-Thr-Asn-Arg

Fig. 8.10

Amberlite 200 peptide separation : lysozyme 2H peptides : concave gradient



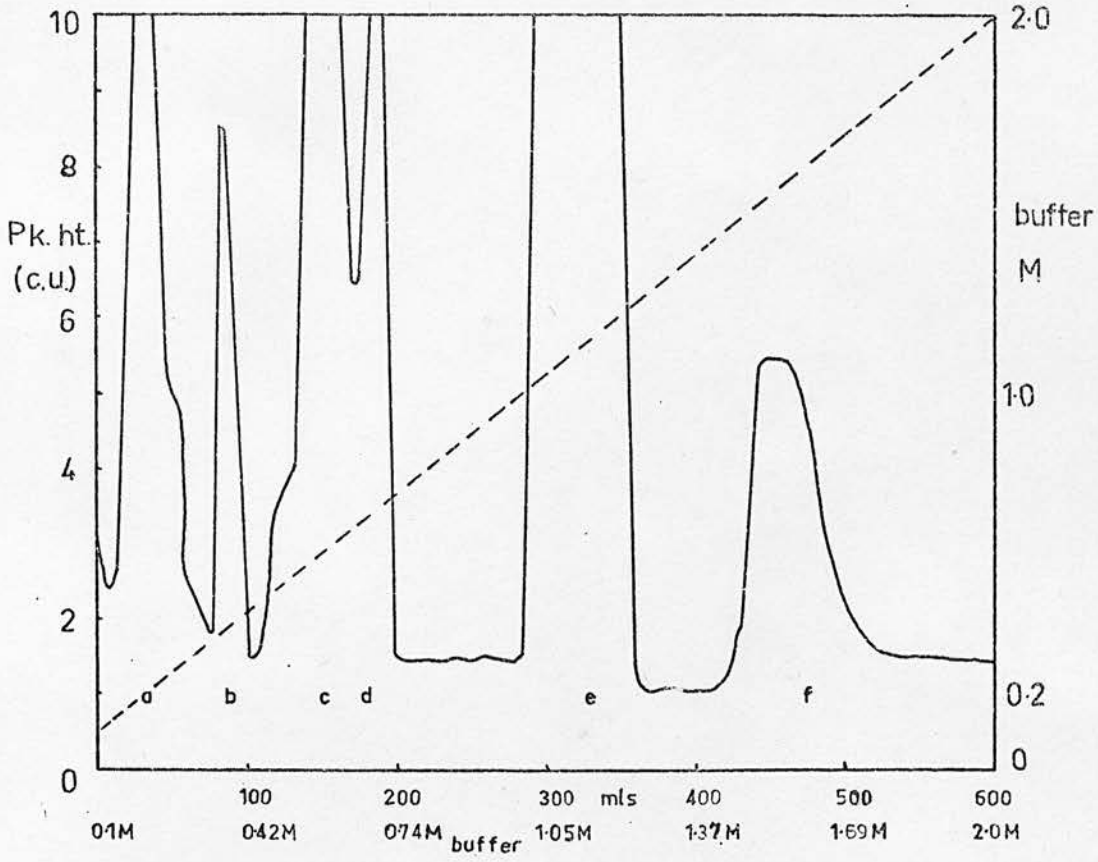
a - T9 ie Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg

b - C_1T9 ie Phe-Glu-Ser-Asn-Phe

c - C_3T9 ie Asn-Thr-Gln-Ala-Thr-Asn-Arg

Fig. 8.11

Amberlite 200 peptide separation : Lysozyme 2JK peptides : linear gradient



a - Void volume mixture

b - T12 ie Asn-Asp-Gly-Arg

c - C_3T10 ie Gln-Ile-Asn-Ser-Arg plus C_1T2 ie Val-Phe

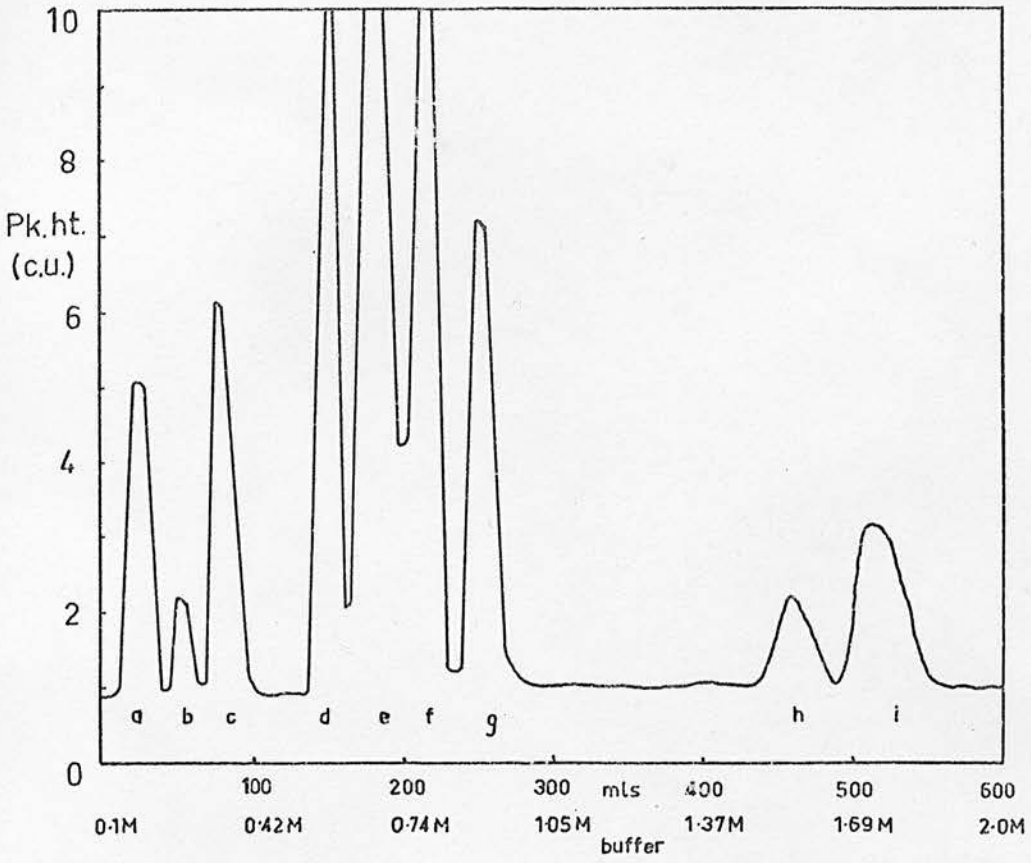
d - C_1T7 ie Gly-Tys plus some T12 ie Asn-Asp-Gly-Arg

e - T24 ie Gly-Ae-Cys plus free Asx

f - T8 ie Ala-Ala-Lys

Fig 8.12

Amberlite 200 peptide separation : Lysozyme 2M peptides : linear gradient



2.0M - 5.0M linear gradient - No peaks

a - T13 ie Thr-Pro-Gly-Ser-Arg

b - trace miscellaneous peptide(s)

c - T20 ie Asn-Arg plus non-characterized peptides

d - C_2T7 ie Ser-Leu-Gly-Asn-Trp

e - T6 ie His-Gly-Leu-Asp-Asn-Tyr-Arg + C_1^1T9 ie Phe

f - T14 ie Asn-Leu-Ae-Cys

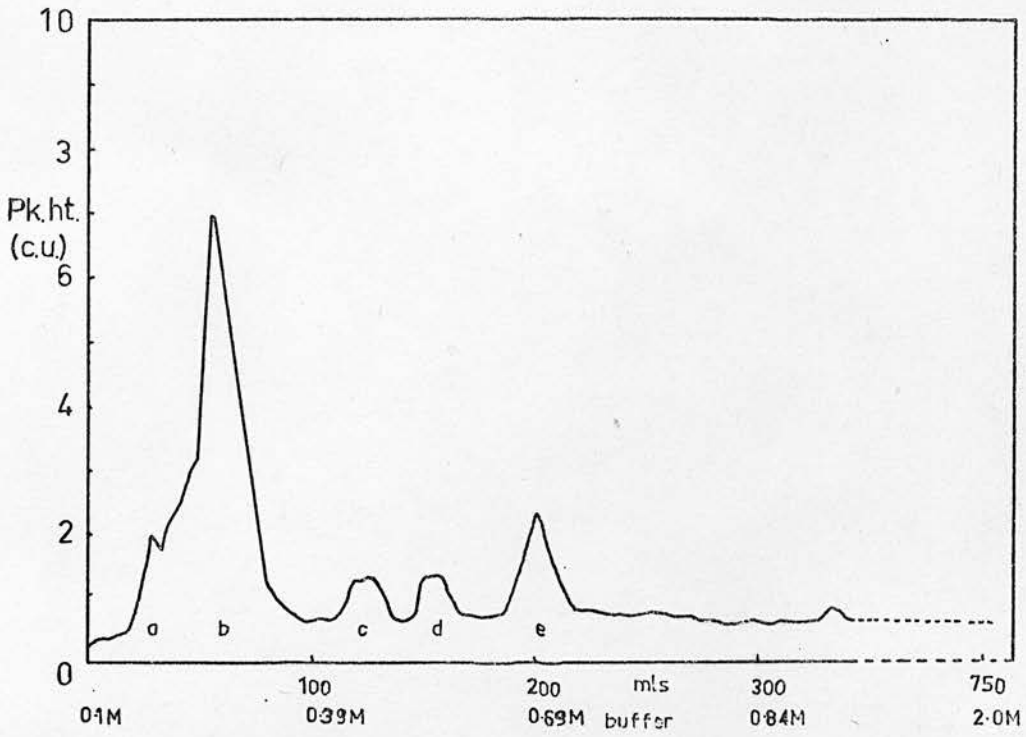
g - T17 ie Ala-Lys

h - non-characterized peptides

i - T24 ie Gly-Ae-Cys

Fig. 8.13

Amberlite 200 peptide separation : lysozyme 3BC peptides : Concave gradient



a - C_1T10 plus a trace of C_1T19 ie Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp plus miscellaneous peptide material

b - C_1T10 ie Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

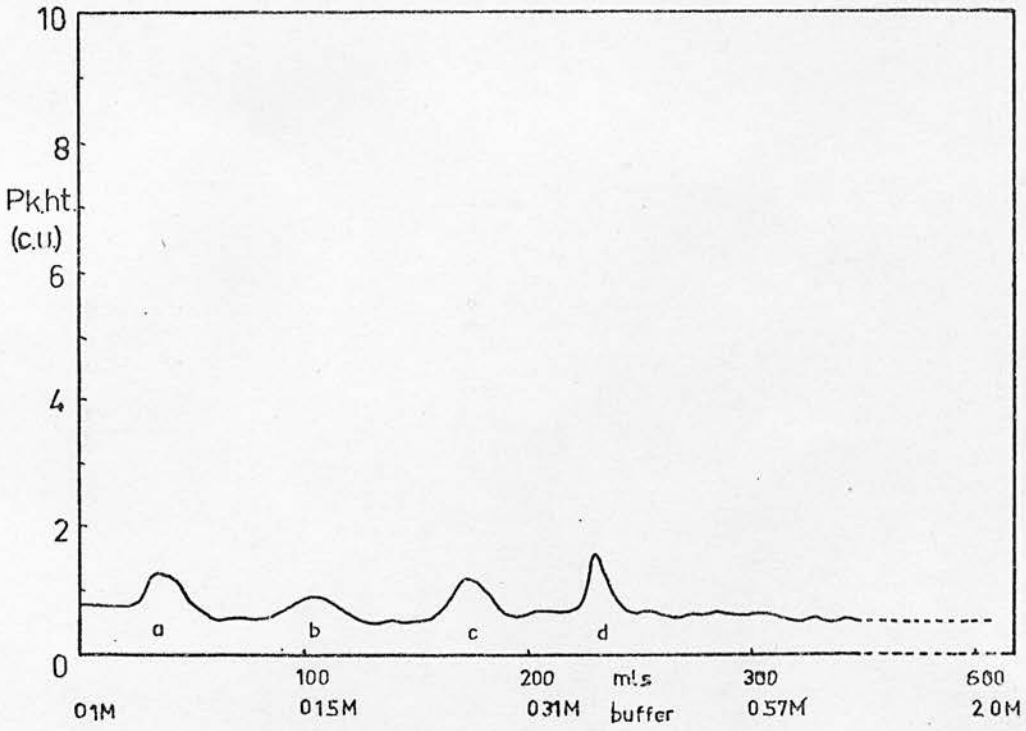
c - Mixture of T16 and T19 peptides

d - Mixture, possibly including CT23 ie Gly-Thr-Asp-Val-Gln-Ala-Trp

e - C_1T4 ie Glu-Leu-Ala-Ala-Ala-Met

Fig. 8.14

Amberlite 200 peptide separation : bovine globin β-chain peptide 1I :
Concave gradient



a - Void volume mixture

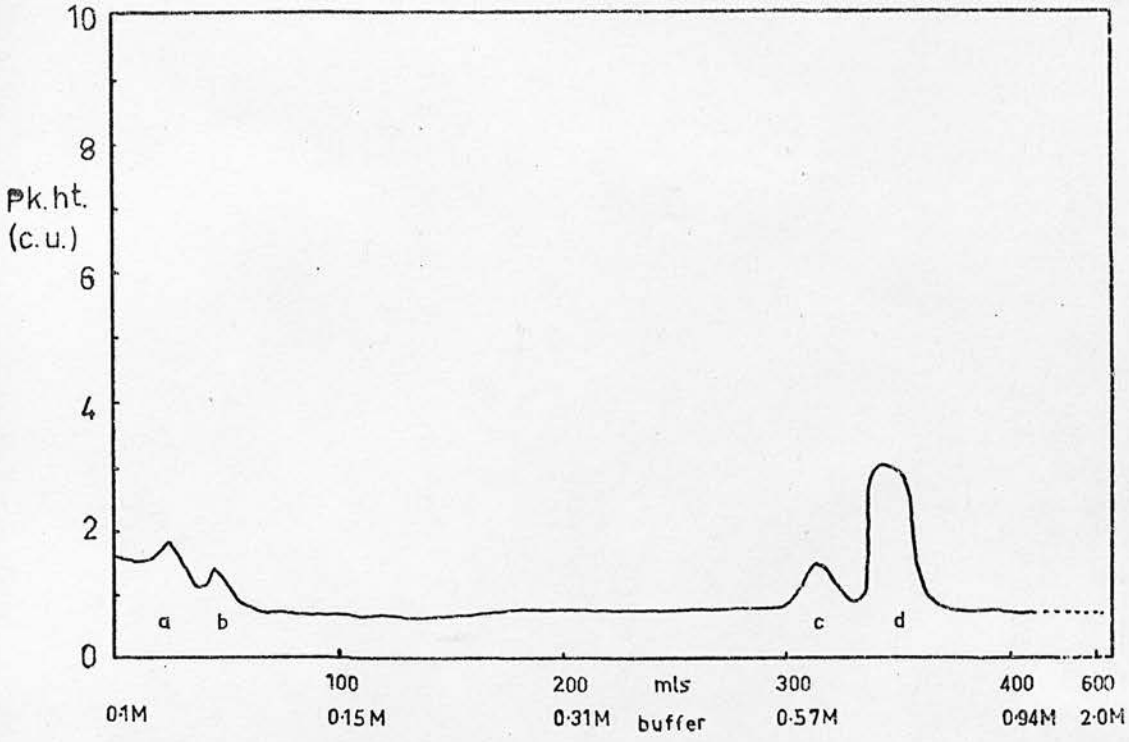
b - trace miscellaneous peptides

c - C_1T11 ie Gly-Thr-Phe

d - C_2T9 ie Ser-Asp-Gly-Met-Lys

Fig. 8.15

Amberlite 200 peptide separation : bovine globin β-chain 2I : Concave gradient



a - Void volume mixture including C_3T5 ie (Asp,Asn,Pro)Lys

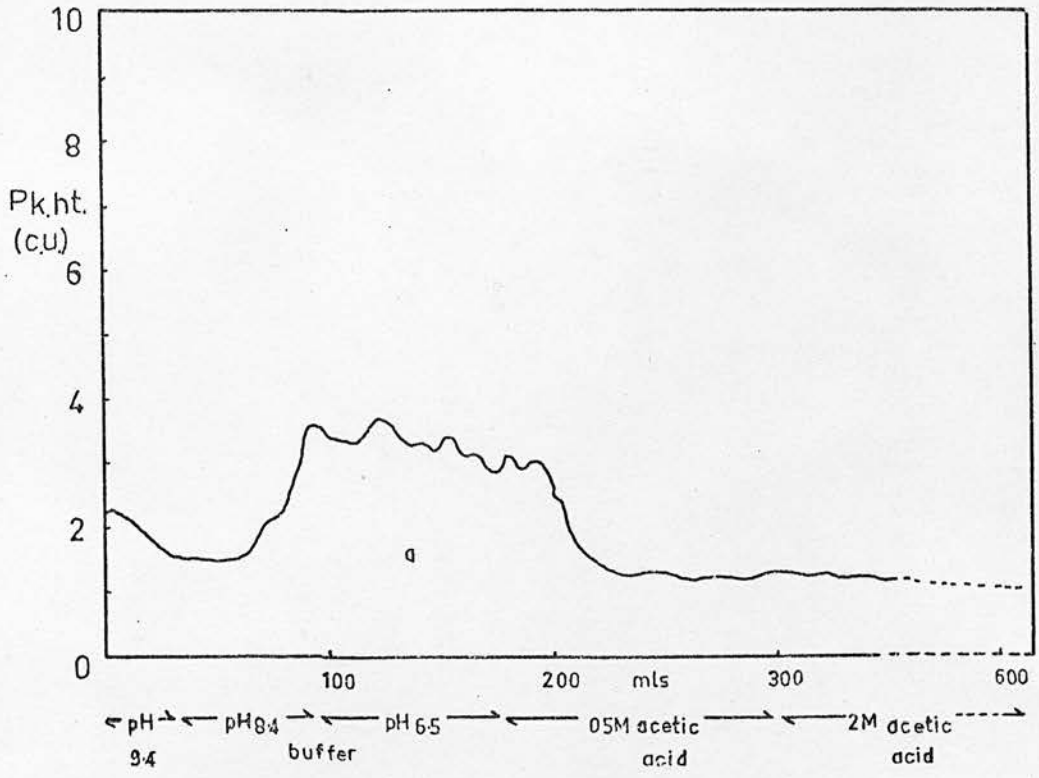
b - Mixture, including C_3T5 ie (Asp,Asn,Pro)Lys

c - T12 ie (Leu-His-Val-Asp-Pro-Glu-Asn-Phe)Lys

d - T3ab ie Val-Lys-Val-Asp-Glu-Val-Gly-Glu-Ala(Leu,Gly)Arg

Fig. 8.16

"Deacidite" FFIP peptide separation : Lysozyme 1Aa peptide : Stepwise buffer change



The column was finally washed with 2M formic acid

a - miscellaneous peptide material

Fig. 8.17

Sequence of Ae-Lysozyme

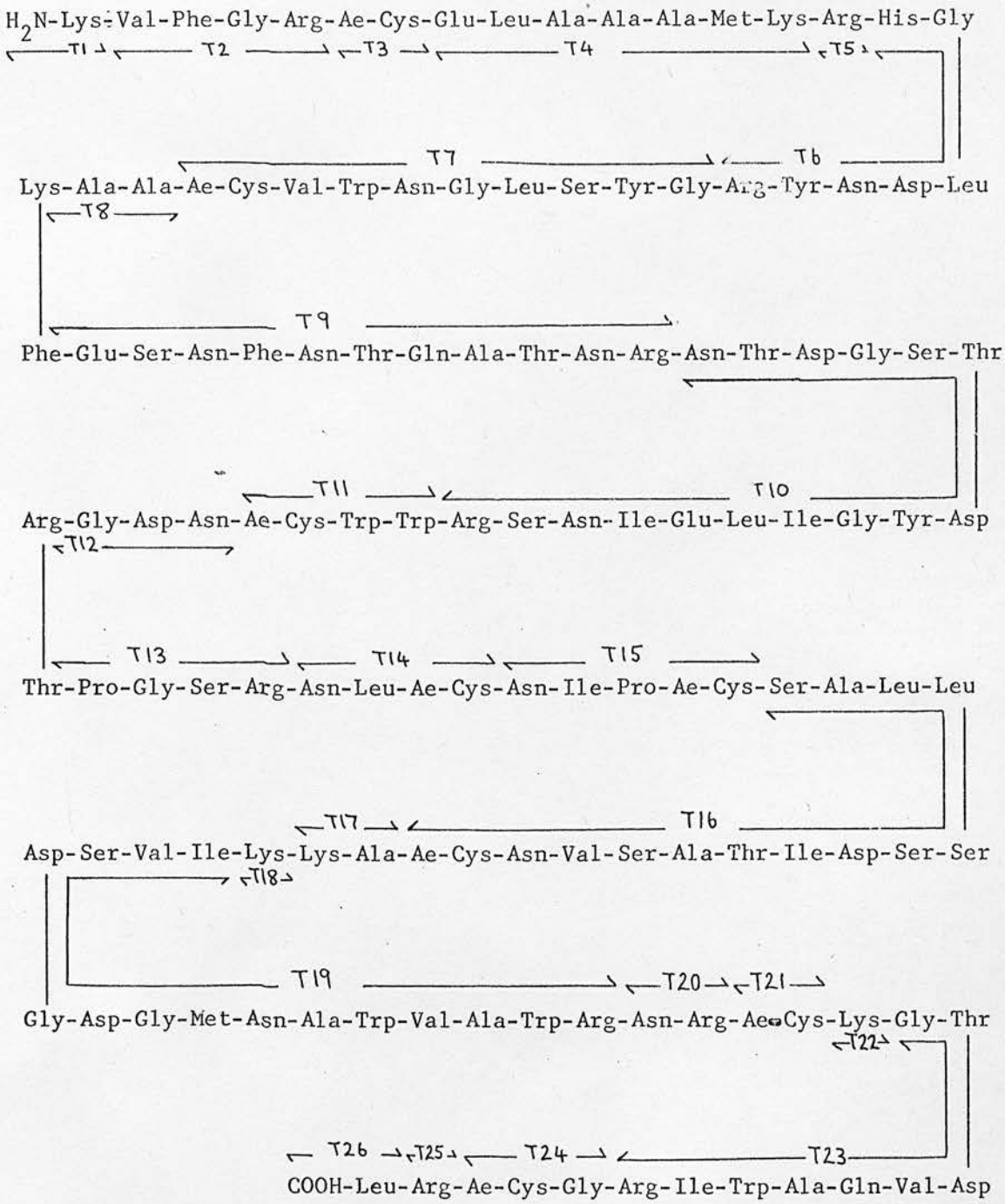


Fig. 8.18

Sequence of β-Ae-bovine globin β_A-Chain

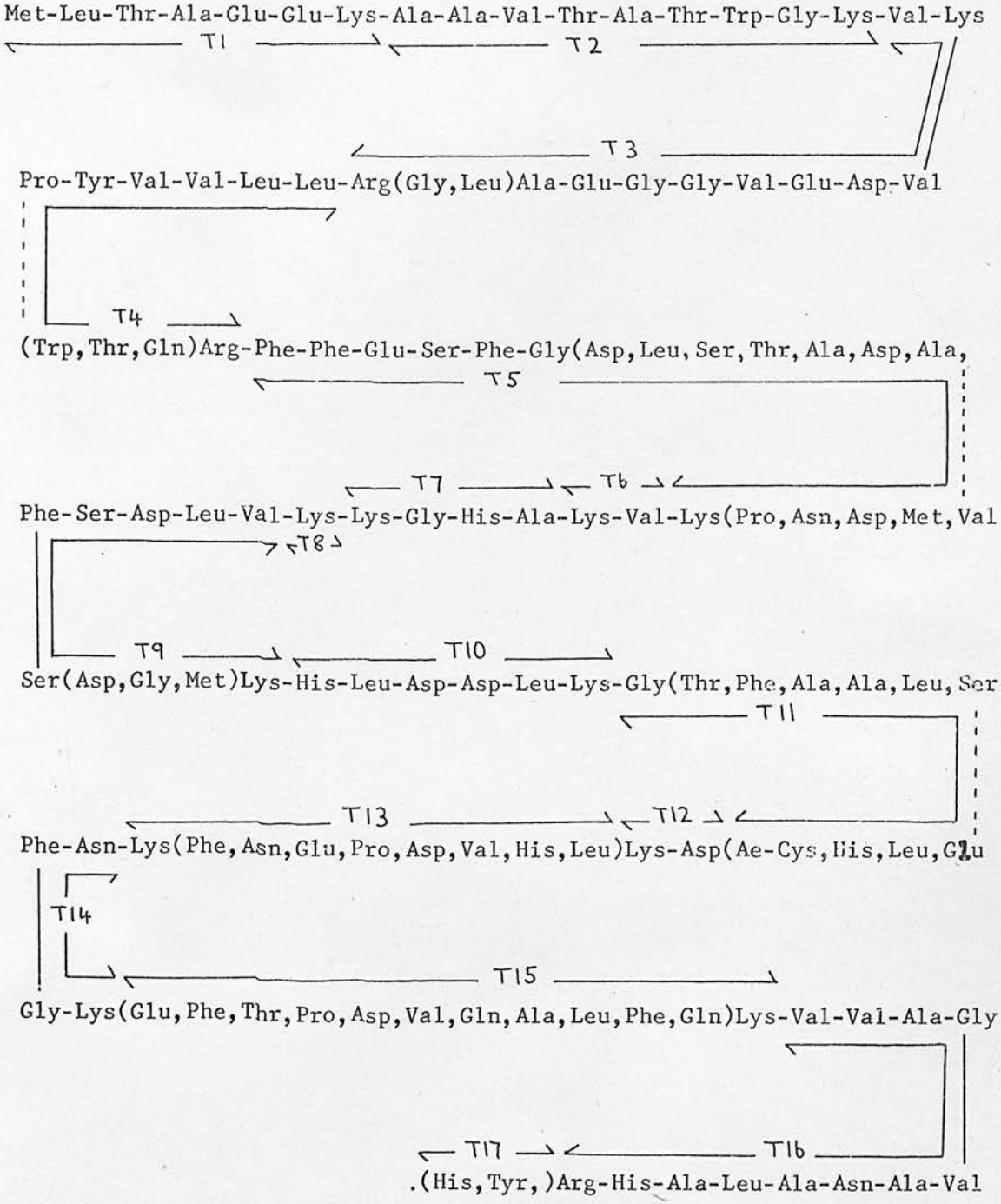


Fig. 8.19

Sequence of β-Ae-bovine globin β_B-Chain

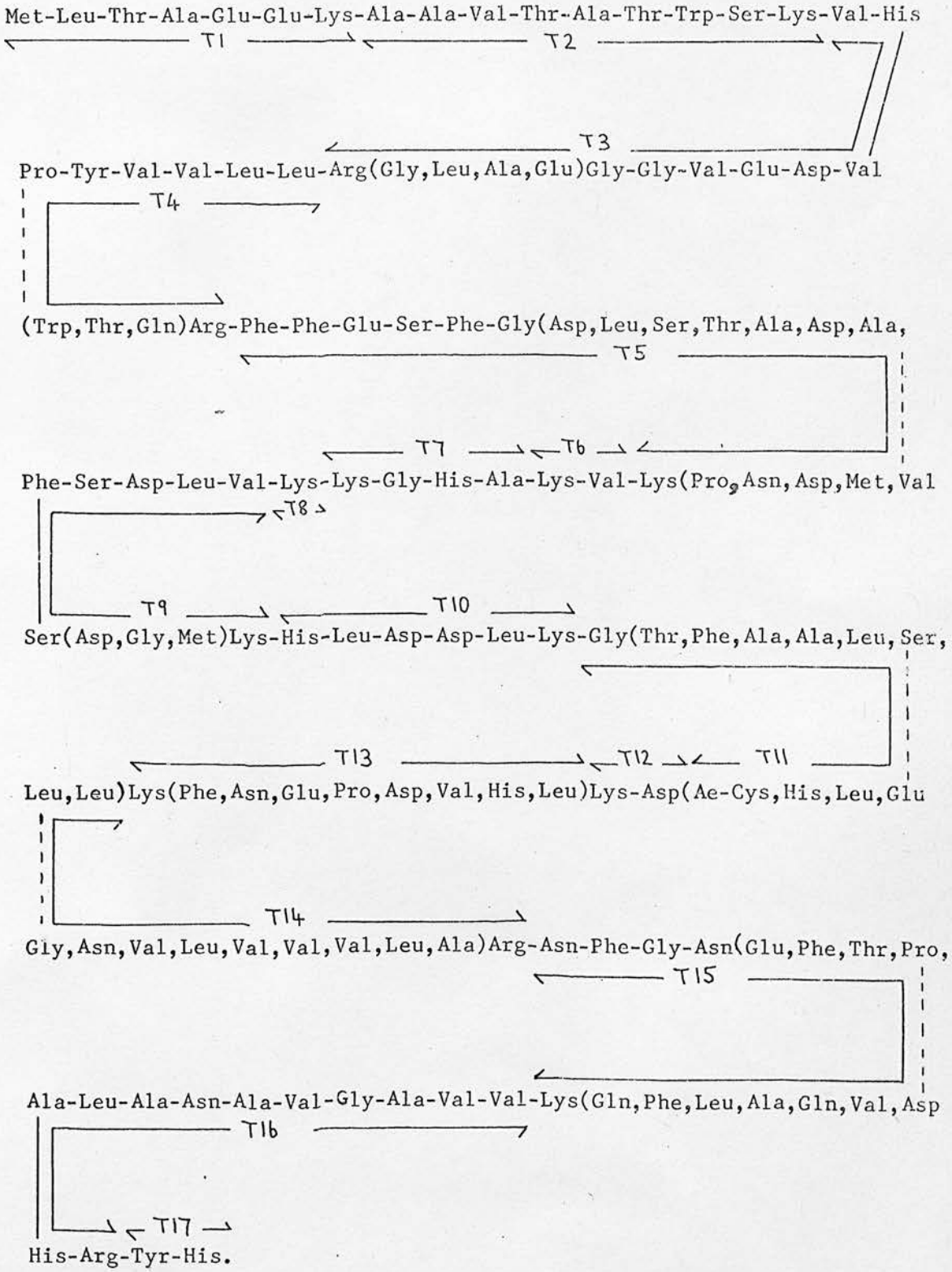


Table 8.4

Order of the Lysozyme Peptides off the Zeo-Karb 225 8% Column and Amberlite 200 Column

Lysozyme 2

Fraction B	C_2T16	-	Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Ae-Cys (10-n)
D	C_1T10	-	Asn-Thr-Asn-Gly-Ser-Thr-Asp-Tyr (8-2a)
E	C_1T19	-	Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp (11-1a)
	C_1T4*	-	Glu-Leu-Ala-Ala-Ala-Met (6-1a)
	T16*	-	Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-Ae-Cys (14-n)
G	C_1T16	-	Ser-Ala-Leu-Leu (4-n)
	C_1T23	-	Gly-Thr-Asp-Val-Gln-Ala-Trp (7-n)
	C_3T7	-	Gly-Asn-Trp (3-1b)
	T9	-	Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg (12-n)
H	C_1T9	-	Phe-Glu-Ser-Asn-Phe (5-1a)
	C_3T9	-	Asn-Thr-Gln-Ala-Thr-Asn-Arg (7-1b)
I	T15	-	Asn-Ile-Pro-Ae-Cys (4-1b)
JK	T12	-	Asn-Asp-Gly-Arg (4-n)
	C_3T10	-	Gln-Ile-Asn-Ser-Arg (5-1b)
	C_1T7	-	Gly-Tyr (2-n)
	T8	-	Ala-Ala-Lys (3-1b)
	$C_3T11,12$	-	Ae-Cys-Asn-Asp-Gly-Arg (5-16)
L	T4	-	Glu-Leu-Ala-Ala-Ala-Met-Lys (7-n)
	C_2T7	-	Ser-Leu-Gly-Asn-Trp (5-1b)
	T24	-	Gly-Ae-Cys (2-1b)

Table 8.4 (Contd.)

Fraction M	T13	-	Thr-Pro-Gly-Ser-Arg (5-1b)
	T20	-	Asn-Arg (2-1b)
	T6	-	His-Gly-Leu-Asp-Asn-Tyr (6-n)
	T14	-	Asn-Leu-Ae-Cys (3-1b)
	T17	-	Ala-Lys (2-1b)

* Theoretical positions from Lysozyme 1 or 3 results

(10-n) ie 10 residue peptide - net acidity neutral

(8-2a) ie 8 residue peptide - net acidity - 2 residues

Table 8.5

Order of the β-chain Peptides off the Zeo-Karb 225 8% Column and the Amberlite 200 Column

Fraction β2F	(	β^{B}T14 - Asn-Phe-Gly-Asn-(Glu,Thr,Pro,Asp,Val,Gln,Ala,Leu, Phe,Gln)-Lys (14-3a)
	(	$\beta^{AB}C_1$T9 - Val-Leu-Asp-Ser-Phe (5-1a)
β1H		β^{AB}T1 - Met-Leu-Thr-Ala-Glu-Glu-Lys (7-1a)
β1I	(	$\beta^{AB}C_2$T9 - Ser-Asp-Gly-Met-Lys (5-n)
	(	$\beta^{AB}C_1$T11- Gly-Thr-Phe (3-n)
β2H		$\beta^{B}C_2$T2 - Ser-Lys (2-1b)
β2I	(	$\beta^{AB}C_3$T5 - (Asp,Asn,Pro)-Lys (4-n)
	(	β^{AB}T12 - (Leu,His,Val,Asp,Pro,Glu,Asn,Phe)-Lys (9-n)
	(	β^{AB}T3ab - Val-Lys-Asp-Glu-Val-Gly-Glu-Ala(Leu,Gly)Arg (12-1a)

Leucine Aminopeptidase Digestion

When samples of peptide which were required for further investigation were obtained in a reasonably pure form they were subjected to a kinetic LAP digestion. Not only did this confirm the sequence of the peptide but it also indicated the degree to which the aspartic acid under study had rearranged to the β-form. β-aspartyl links are largely resistant to the action of LAP.[37] The LAP results were compared with the amino acid analysis of the substrate so as to more easily neglect interference from traces of peptide impurities.

The first peptide to be digested was $Lyl C_1 T10$. The amino acid analysis and LAP analysis are shown in table 10.3. From the amino acid analysis it would appear that there are some impurities in the peptide but that there is not an excess of aspartic acid. Aspartic acid was not observed in the LAP run until after 8 hours and by this time the presence of tyrosine was observed. The two other tyrosine containing peptides had already been found and since this amino acid is not a common constituent of lysozyme it would appear that all the tyrosine was, in fact, coming from this peptide. After 48 hours the amount of aspartic acid present is the same as that of tyrosine present. It is postulated that the αβ-Asp-Gly (or β-Asp-Gly) is being cleaved off the chain as a dipeptide to produce a peptide N-terminal in serine. αβ-Asp-Gly is a compact dipeptide. The LAP could then continue along the chain cleaving off residues until it reached C-terminal tyrosine. For this theory to hold however all the glycine observed would have to come from impurities. The glycine content in the amino acid analysis was high and glycine is a frequent contaminant in peptide chromatograms.

If αβ-Asp-Gly was being cleaved from the peptide and converting to β-Asp-Gly under the basic conditions of digestion then a peak corresponding to β-Asp-Gly would be expected. A small broad peak was observed emerging before glycine in all mixtures of the 2-48 hour run except for the controls.

Two peaks were observed in the early part of the chromatogram before aspartic acid. The latter peak (a double peak) is caused by the N-methylmorpholine digestion buffer. It was observed that the first peak, which had a retention time of 35 minutes compared with 68 minutes for aspartic acid, increased in size with LAP digest time; it had a peak area of 2.75 at t = 8 hours and one of 7.18 at t = 48 hours. Using the separation conditions of Dorer,[25] ie sodium phosphate buffer, 0.5 M in sodium and pH 1.82, β-aspartyl-glycine is eluted before aspartic acid and much earlier than α-aspartyl-glycine. However using the pH 3.28 sodium citrate buffer this degree of separation would not be expected and the peak around glycine is more likely to represent β-Asp-Gly. The early peak is possibly on LAP breakdown product.

The reason for the initial high level of asparagine in the 'long term' run followed by a fall is possibly due to a peptide being eluted with asparagine in the initial stages. The value for asparagine starts to fall as those of aspartic acid and tyrosine start to rise, so possibly the peptide is the 6 residue one Asp-Gly-Ser-Thr-Asp-Tyr.

However, whether or not the postulate outlined above is true, the peptide seems to be present largely in the rearranged form.

Another batch of this peptide was collected ($Ly2C_1T10$) and digested with LAP in the same way. The amino acid analysis and LAP analysis are shown in table 10.4. It can be seen that the peptide contains some

impurities, including aspartic acid. Asparagine and threonine were easily cleaved from the peptide and aspartic acid, and more significantly tyrosine, were observed after only 1 hr. 20 minutes.

This batch of the peptide would appear to be largely in the unrearranged form and was used for further study on the rearrangement process.

The supposed tetrapeptide, Asn-Asp-Gly-Arg (see later) was next investigated as sample Ly1T12 and the amino acid analysis and LAP analysis are shown in table 10.5. The peptide impurities present seem to be Val-Ae-cys from T7 and the T24 peptide, Gly-Ae-cys. The asparagine residue was easily removed but the aspartic acid was done so with difficulty and to a much lesser degree. No arginine was cleaved from the peptide. Thus this sample of peptide seems to be in the rearranged state.

The peptide Ly2T12 was next examined and the amino acid analysis and LAP analysis are shown in table 10.6. Asparagine was easily cleaved but aspartic acid was cleaved only to a small degree. The peptide would appear to be a mixture of both rearranged and unrearranged peptide, the former predominating.

Batch 1 lysozyme peptides were concentrated by means of a rotary evaporator and water bath at 40°C. whilst in batch 2, freeze drying was employed to concentrate the peptide. Batch 1 was also digested with trypsin for longer than batch 2 and a 1:50 ratio of enzyme to substrate was used instead of 1:75. Ammonium carbonate (2M) was used as a neutralising agent in the pH-stat. Batch 2 was digested with trypsin as stated in the experimental section.

The peptide Ly2CT19 was digested with LAP but the results were analysed by dansylation of the amino acid/peptide mixture and separation on TLC polyamide plates. The results are shown in table 10.7. Aspartic

acid was first clearly observed after 2 hours but spots corresponding to methionine and tryptophan were not seen until after 48 hours. It would seem that perhaps the first aspartic acid residue is largely unrearranged, but that the second one is largely in the rearranged state. However early DNS-Asp spots may be due to the high level of aspartic acid impurity in the peptide. This peptide was found to be strangely elusive and was always obtained in a mixed state.

The possibility of deamidation of N-terminal asparagine residues has already been discussed[23,25]. In the natural product peptides that were N-terminal in this amino acid, ie $Lyl C_1 T10$, $Ly2 C_1 T10$, Lyl T12, Ly2 T12, the possibility of N-terminal asparagine deamidation must not be overlooked. If it does occur to any extent then the β-aspartyl unit must be formed since the α-form is susceptible to LAP action and no aspartic acid was liberated in the early stages of the runs with $Lyl C_1 T10$ and Lyl T12. It can be said that deamidation does not predominate.

The synthetic peptide, S1, was treated with LAP and the reaction was fast. Asparagine, glycine ethyl ester and a trace of aspartic acid were observed. The degree of cleavage after 8 hours was approximately the same as that after 1 hour. The synthetic peptide was thus in the unrearranged state, and very little, if any, N-terminal deamination had occurred.

Dansyl Edman Results

This technique was used to check the peptide sequence and to ascertain the degree of rearrangement. Although α-aspartyl and asparaginyl peptides are susceptible to Edman degradation, β-aspartyl peptides are not, a repeating DNS-Asp unit being observed on the TLC plate after dansylation. In β-aspartyl residues a thiazinone ring system would have

Table 10.3

Peptide LysC_1T10:- Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

Amino Acid Analysis

	μmoles	Residues		μmoles	Residues		μmoles	Residues
Asp	0.1286	2.7	Val	0.0166	0.35	Lys	-	-
Thr	0.0810	1.7	Met	-	-	As-Cys	-	-
Ser	0.0559	1.2	Ile	0.0147	0.3	His	-	-
Glu	0.0291	0.6	Leu	0.0118	0.25	Arg	-	-
Pro	-	-	Tyr	0.0478	1.0			
Gly	0.0651	1.4	Phe	-	-	Trp	-	-
Ala	0.0200	0.5						

LAP Analysis: Conc. run (ii) ≠ Conc. run (i)

Corrected Peak Areas

	Time	Asp	Thr	Ser	Asn	Gly	Tyr
(i)	10 mins	-	trace	trace	trace	-	-
	20 "	-	0.47	0.24	0.85	trace	-
	40 "	-	0.95	0.66	1.37	0.54	-
	80 "	-	1.35	1.10	1.79	0.59	-
	120 "	-	1.80	1.03	1.86	0.88	-
(ii)	2 h	-	2.31	1.60	3.57	1.51	-
	4 "	-	2.48	1.64	3.66	1.61	-
	8 "	0.23	2.57	2.06	3.11	1.91	0.62
	24 "	1.69	3.75	2.56	2.78	2.78	0.72
	48 "	2.01	5.55	4.88	5.35	5.36	2.13

Table 10.4

Peptide $Ly2C_1T10$:- Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

Amino Acid Analysis

	μmoles	Residues		μmoles	Residues		μmoles	Residues
Asp	0.0802	3.1	Val	0.0062	0.23	Lys	-	-
Thr	0.0462	1.7	Met	-	-	Ae-Cys	-	-
Ser	0.0323	1.2	Ile	-	-	His	-	-
Glu	0.0071	0.26	Leu	-	-	Arg	-	-
Pro	-	-	Tyr	0.0213	0.8			
Gly	0.0337	1.5	Phe	-	-	Trp	·	·
Ala	0.0082	0.3						

LAP Analysis

Corrected Peak Areas

Time	Asp	Thr	Ser	Asn	Gly	Tyr
20 min.	1.68	6.68	1.51	16.57	1.65	-
1 h.20 mins.	2.81	17.94	3.31	16.41	2.51	0.26
2 h.10 mins.	2.52	18.68	5.39	19.31	3.04	0.75
4 h. 5 mins.	6.51	22.51	6.22	19.53	4.16	0.94
6 h.	7.12	25.63	7.21	21.78	4.76	1.81
24 h.	14.52	25.72	8.74	33.41	5.33	2.93

Table 10.5

Peptide Lyl T12 or C_3T11,12 - Asn-Asp-Gly-Arg or Ae-Cys-Asn-Asp-Gly-Arg
(See Mass Spectrometry results)

Amino Acid Analysis

	μmoles	Residues		μmoles	Residues		μmoles	Residues
Asp	0.0275	1.7	Val	0.0057	(½)	Lys	-	-
Thr	-	-	Met	-	-	Ae-Cys	0.0102	(1)
Ser	0.0097	(1)	Ile	-	-	His	-	-
Glu	-	-	Leu	-	-	Arg	0.0151	0.94
Pro	-	-	Tyr	-	-			
Gly	0.0219	1.35	Phe	-	-	Trp	·	·
Ala	-	-						

LAP Analysis

	Corrected Peak Areas		
Time(h)	Asp	Asn	Gly
2	0	0.62	0.29
4	0	0.68	0.33
24	0.16	1.18	0.53
48	0.19	1.34	0.85

A peak for β-aminoethylcysteine was also observed

No Arginine was observed

Table 10.6

Peptide Ly2T12:- Asn-Asp-Gly-Arg

Amino Acid Analysis

	μmoles	Residues		μmoles	Residues		μmoles	Residues
Asp	0.0199	1.8	Val	0.0030	0.3	Lys	-	-
Thr	-	-	Met	-	-	Ae-Cys	0.0022	0.2
Ser	0.0066	0.6	Ile	0.0028	0.25	His	-	-
Glu	0.0045	0.4	Leu	-	-	Arg	0.0089	0.8
Pro	-	-	Tyr	-	-			
Gly	0.0151	1.4	Phe	-	-	Trp	·	·
Ala	-							

LAP Analysis

	Corrected Peak Areas		
Time(h)	Asp	Asn	Gly
2	0.07	0.38	0.03
24	0.07	0.40	0.07

to replace the thiazolinone ring.

Peptide Ly1C_1T10 was subjected to a 5-tube Edman degradation followed by dansylation to observe the first five residues from the N-terminus. The results are shown in fig. 10.5. No glycine was observed in tube 4 or serine in tube 5 but faint spots corresponding to DNS-Asp were observed in positions corresponding to both these tubes. Tube 3 was shown to contain DNS-Asp, tube 2 DNS-Thr but no spot at all was seen for tube 1. Tests with standards had shown that the method was not as sensitive for asparagine as it was for most of the other amino acids. These results confirm those of the LAP digest, the peptide is in the rearranged state.

The experiment was repeated with peptide Ly2C_1T10 (fig.10.6). Nothing was observed in tube 1 but tube 2 was seen to contain DNS-Thr. Tube 3 contained DNS-Asp and tube 4 both DNS-Asp and DNS-Gly. Tube 5 was lost and tube 6 contained DNS-Thr and a faint streak around the DNS-Asp position. It would appear that both forms of aspartyl are present with the unrearranged form predominating. This agrees with the LAP results.

A third batch of this peptide was tested (Ly3C_1T10) and the amino acid analysis and results are shown in table 10.8. The results showed that this batch of the peptide was in the largely unrearranged state since the major spot in tube 4 was DNS-Gly and DNS-Ser was observed in tube 5.

The tetrapeptide Ly2T12 was investigated and found to be a mixture of both rearranged and unrearranged peptides (fig. 10.7) DNS-Gly was observed in tube 3 and a faint spot in the position corresponding to DNS-Arg was noted. A little DNS-Asp was observed in tubes 3 and 4

as well as a good spot in tube 2. Tube 1 indicated the presence of DNS-Asn and also a trace of DNS-Asp. This may be an impurity since a little DNS-Gly was also observed but it may indicate that N-terminal asparagine deamidation had taken place to some extent. The LAP results suggested that the peptide was in the rearranged form but the Dansyl-Edman results indicated that a mixture was present.

The β-chain bovine globin peptide $\beta 1C_2T9$ was next treated and the amino acid analysis and results are shown in table 10.7. In tube 3 the spot corresponding to DNS-Asp was stronger than that for DNS-Gly indicating yet again the presence of a mixture of rearranged and unrearranged forms.

Finally a run was done with a peptide that did not contain an Asp-Gly or an Asn-Gly sequence but an Asn-Trp sequence. It was $Ly2C_2T7$ - Ser-Leu-Gly-Asn-Trp (fig. 10.8). DNS-Ser, DNS-Leu and DNS-Gly were located in tubes 1, 2 and 3 respectively. No spot was obtained in tube 4 and in tube 5 a single strong amino acid spot corresponding to that for DNS-Trp was observed. The fact that no DNS-Asp was observed in 4 or 5 and the presence of DNS-Trp in tube 5 indicates that the asparagine next to tryptophan is not rearranged at all.

Mass Spectrometry

Mass spectrometry of peptides is a relatively new sequencing technique. The principal points of fragmentation are the bonds on either side of the carbonyl group, i.e.

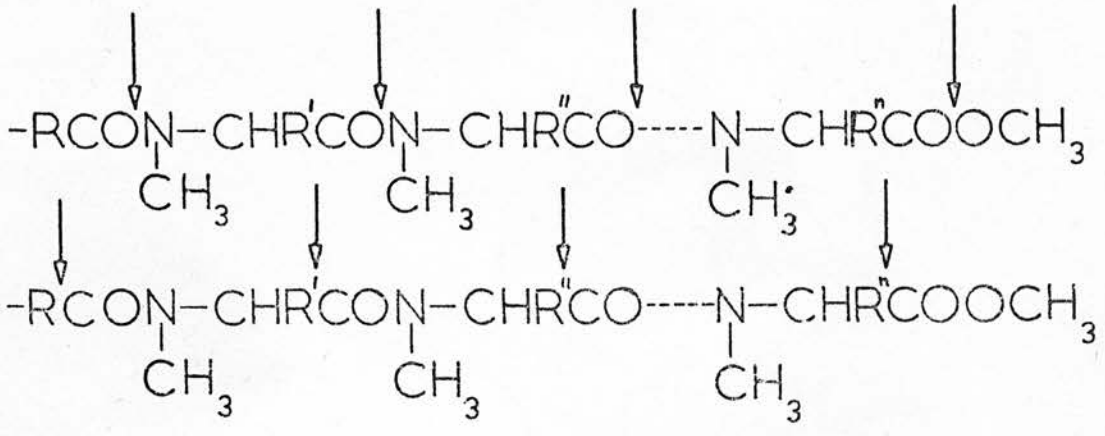


Table 10.7

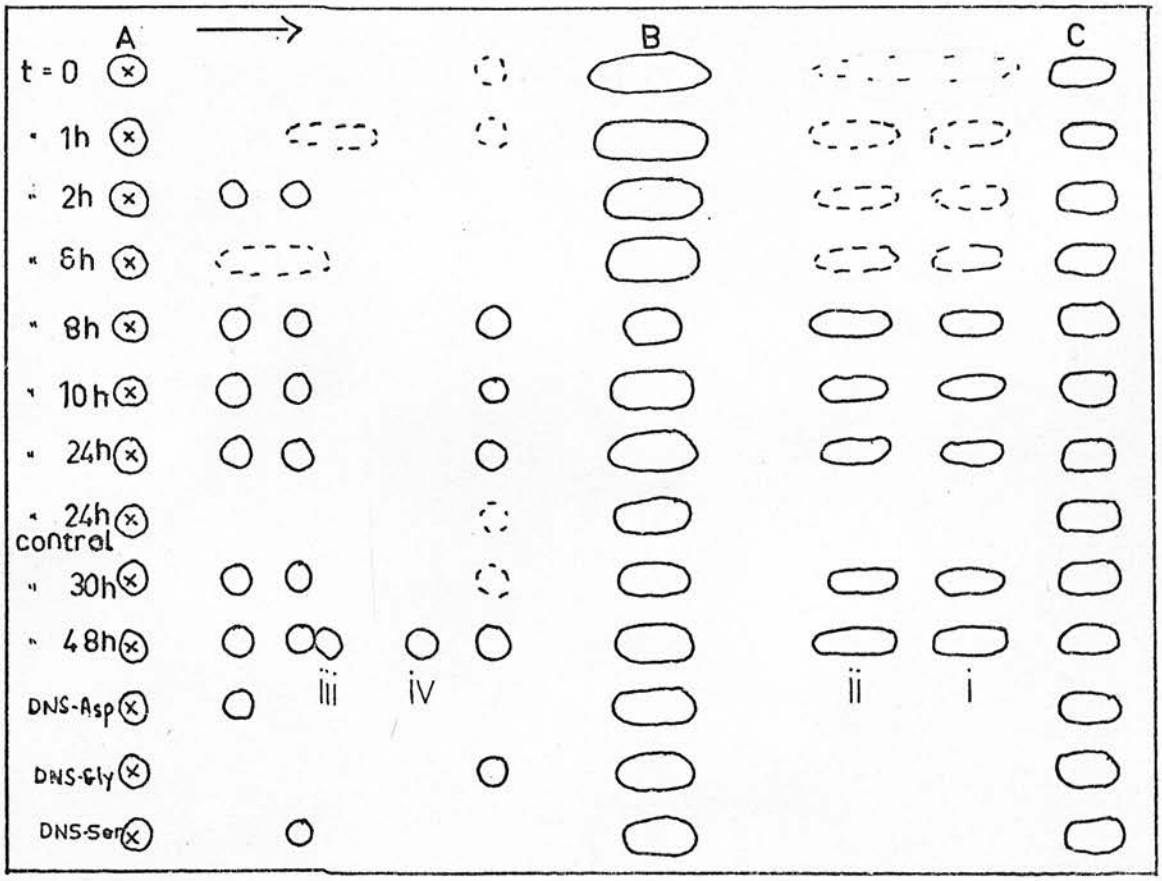
Peptide Ly2CT19:- Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp (impure)

Amino Acid Analysis

	µmoles	Residues		µmoles	Residues		µmoles	Residues
Asp	0.0584	6.0	Val	0.0097	1.0	Lys	-	-
Thr	0.0126	1.3	Met	0.0052	0.54	Ae-Cys	-	-
Ser	0.0359	3.7	Ile	0.0088	0.91	His	-	-
Glu	-	-	Leu	0.0074	0.76	Arg	-	-
Pro	-	-	Tyr	0.0023	0.24			
Gly	0.0332	3.4	Phe	-	-	Trp		
Ala	0.0172	1.8						

LAP - DANSYLATION ANALYSIS

TLC:- Stationary phase - polyamide: Solvent-benzene/Acetic Acid, 9:1



In the 48 hr. run spot (i) corresponds to that for DNS-Ile, spot (ii) that for DNS-Val, spot (iii) for DNS-Met and (iv) for DNS-Trp; this information was obtained from literature separations of all the dansyl amino acids.

Spots A - Correspond to DNS-OH and were blue in colour

Spots B " " $DNS-NH_2$ and were blue in colour

Spots C " " dansyl chloride breakdown products.

Table 10.8

Peptide $Ly3C_1T10$:- Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

Amino Acid Analysis

	µmoles	Residues		µmoles	Residues		µmoles	Residues
Asp	0.1911	3.1	Val	0.0177	0.29	Lys	-	-
Thr	0.1231	2.0	Met	0.0149	0.25	Ae-Cys	-	-
Ser	0.868	1.4	Ile	0.0160	0.26	His	-	-
Glu	0.0314	0.5	Leu	0.0193	0.31	Arg	0.0096	0.15
Pro	-	-	Tyr	0.0497	0.8			
Gly	0.0893	1.4	Phe	-	-	Trp	.	.
Ala	0.0544	0.9						

Dansyl Edman Results

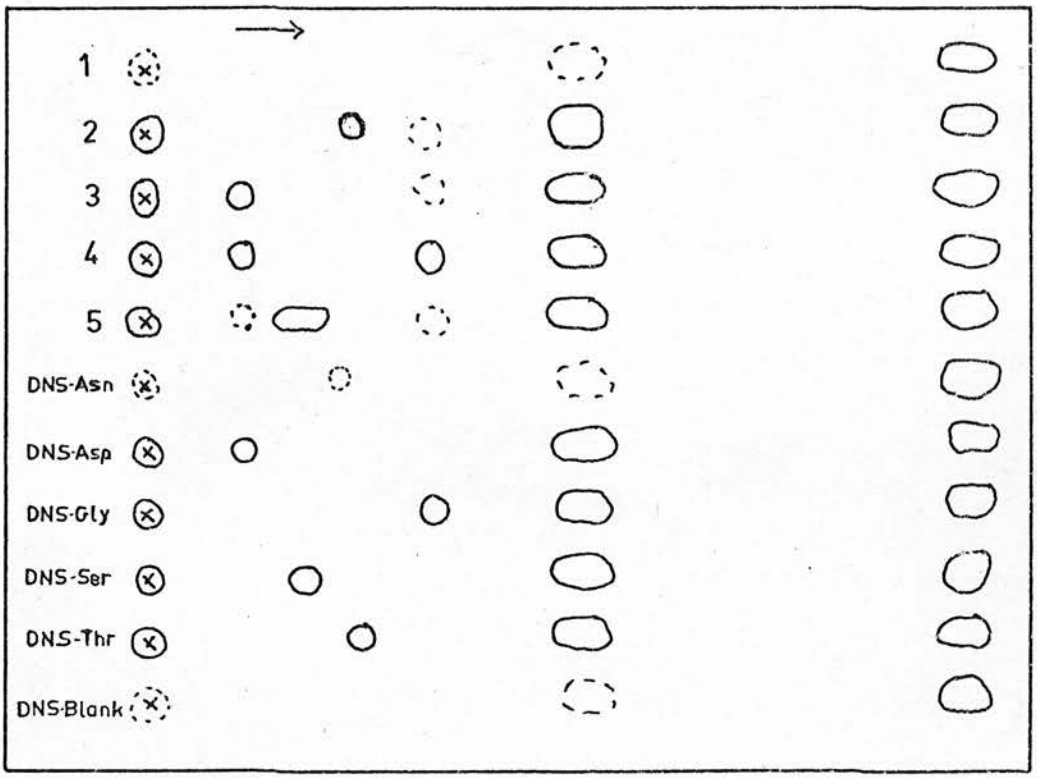


Table 10.9

Peptide $\beta 1C_2T9$:- Ser-Asp-Gly-Met-Lys

Amino Acid Analysis

	µmoles	Residues		µmoles	Residues		µmoles	Residues
Asp	0.0096	1.4	Val	-	-	Lys	0.0063	1
Thr	-	-	Met	trace		Ae-Cys	-	-
Ser	0.0069	1	Ile	-	-	His	-	-
Glu	0.0070	1	Leu	-	-	Arg	-	-
Pro	-	-	Tyr	-	-			
Gly	0.0108	1.5	Phe	-	-	Trp	.	.
Ala	0.0108	1.5						

Dansyl Edman Analysis

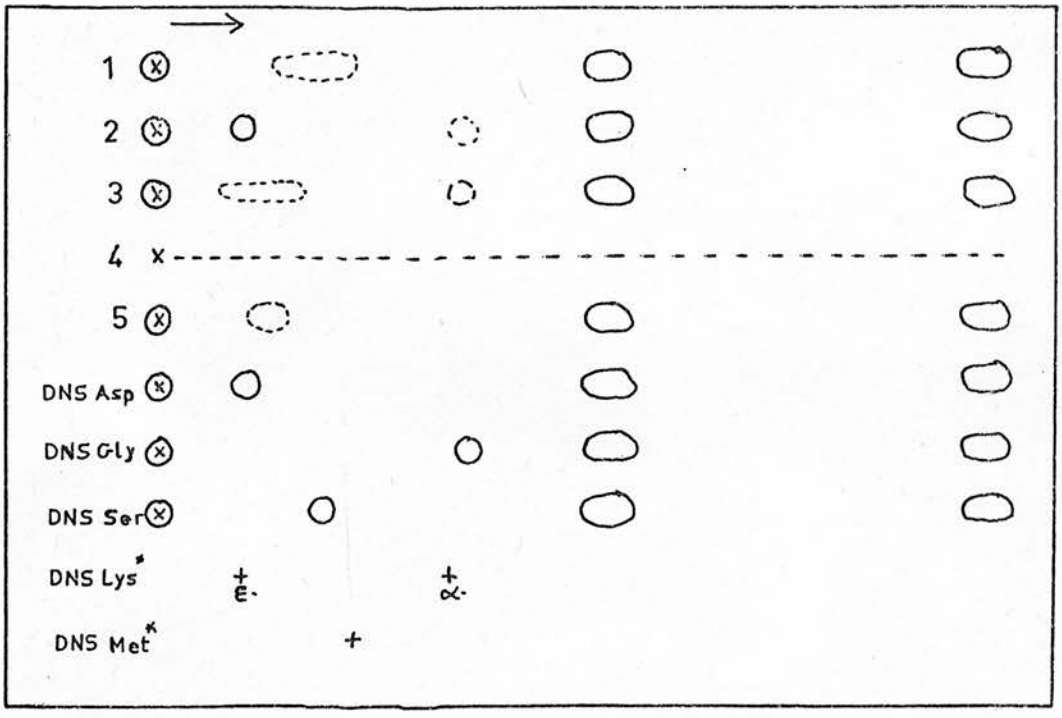
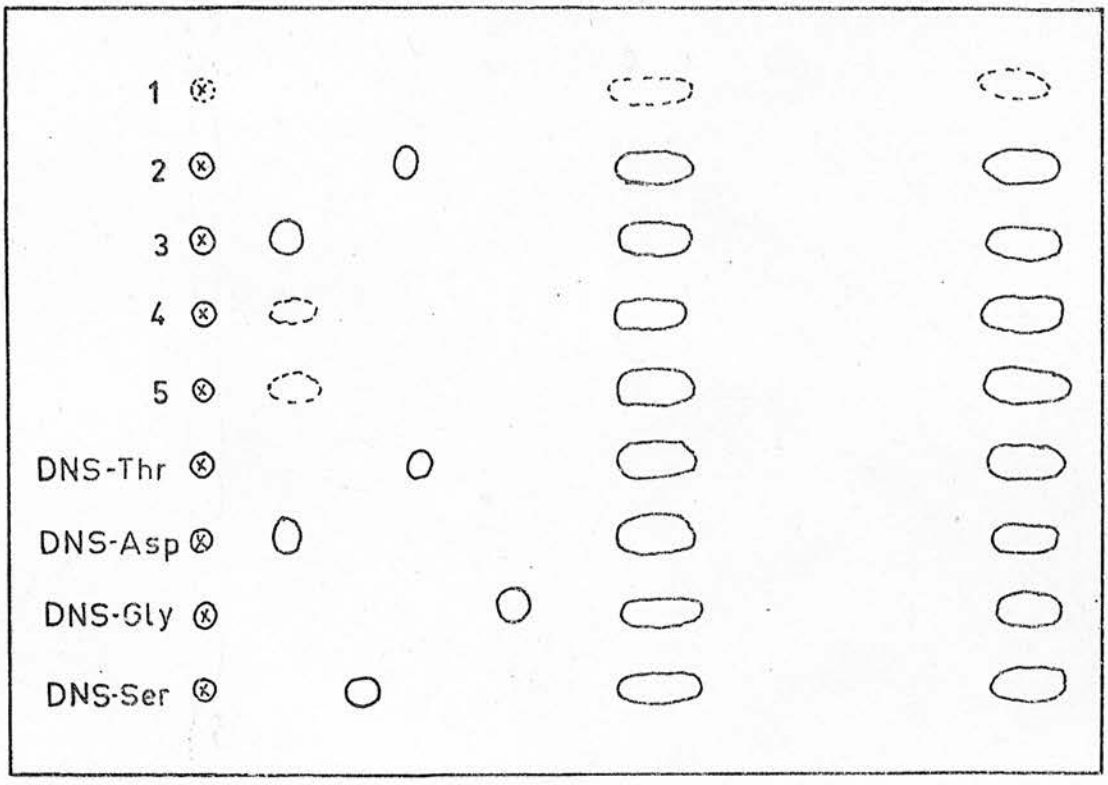


Fig.10.5 Dansyl - Edman: Ly1C,T10



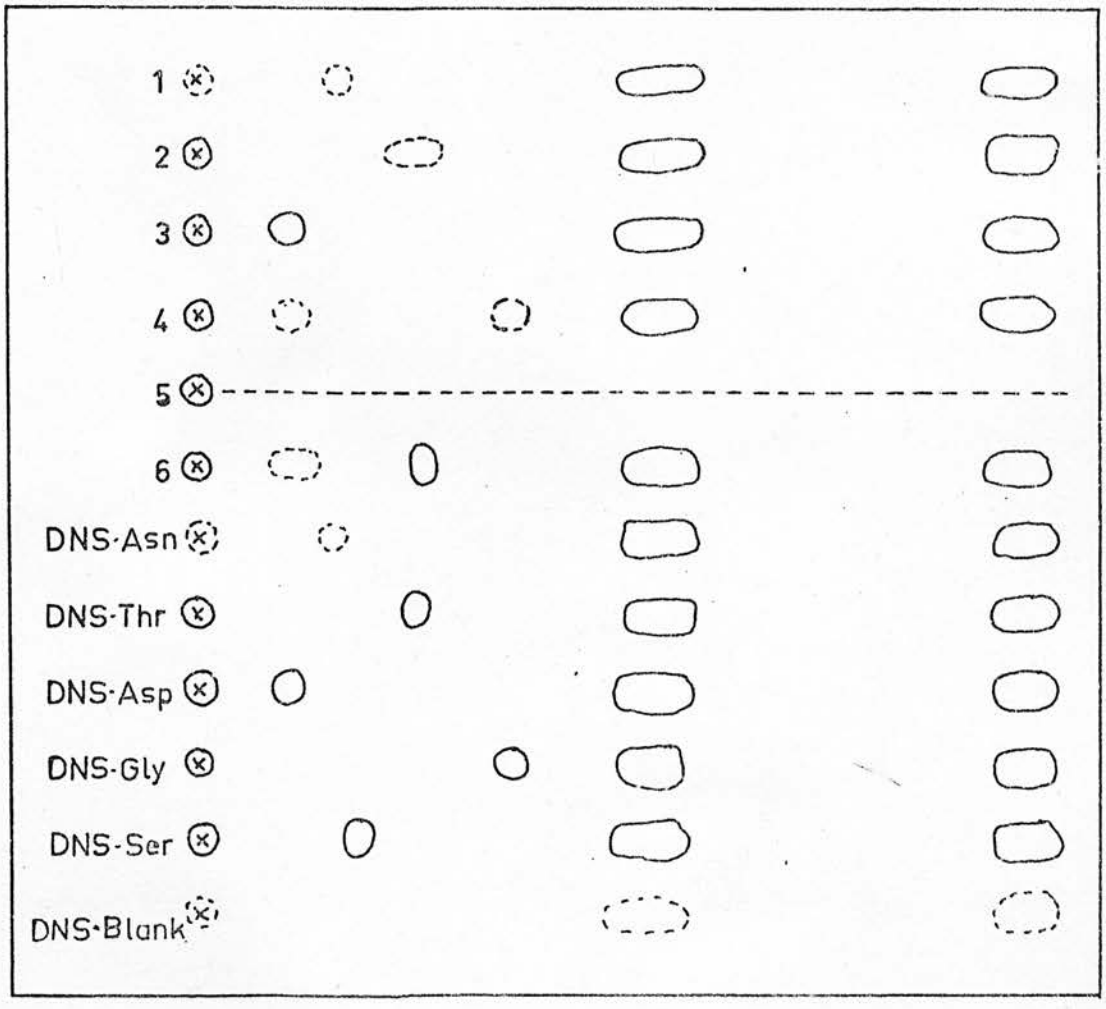


Fig.10.6 Dansyl-Edman: Ly 2 C,T 10

Fig.10.7 Dansyl-Edman: Ly2T12

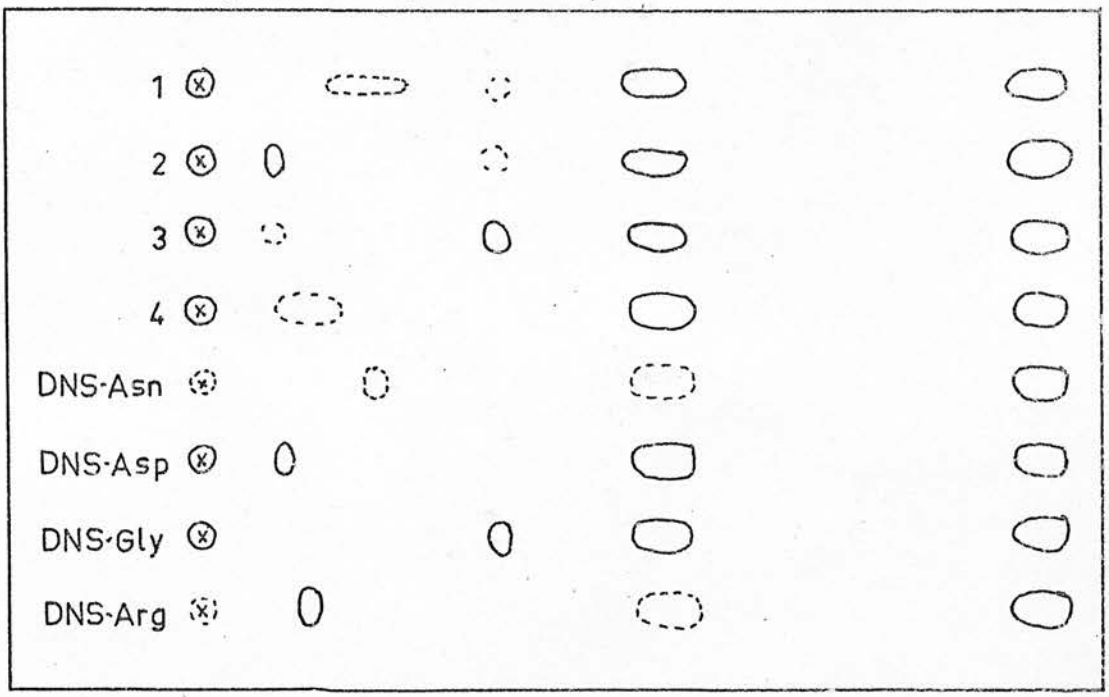
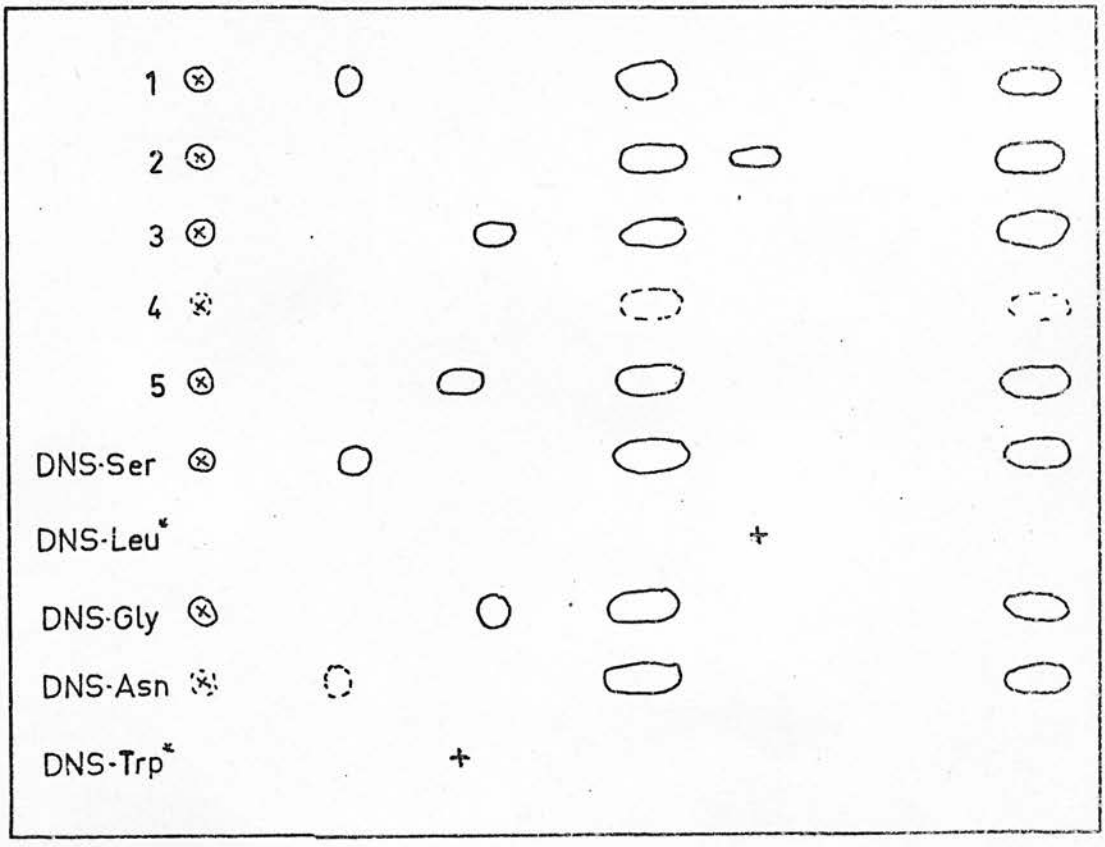


Fig.10.8 Dansyl-Edman: Ly2CT7



* - lit. position

In addition there is a tendency for the side chains of valine, leucine, asparagine, serine, threonine and cysteine to be lost. Amino acid residues are lost from the C-terminus and the sequence is built up from the N-terminus.

Peptide LylC_1T10, which has the sequence Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr, was derivatised as described in the experimental section and its mass spectrum recorded at a probe temperature of 300°C. The following sequence peaks were expected.

Asn - $\frac{m}{e}$ 199

Asn-Thr - $\frac{m}{e}$ 328

Asn-Thr-Asp - $\frac{m}{e}$ 471

Asn-Thr-Asp-Gly - $\frac{m}{e}$ 542

Asn-Thr-Asp-Gly-Ser - $\frac{m}{e}$ 657

Asn-Thr-Asp-Gly-Ser-Thr - $\frac{m}{e}$ 786

Asn-Thr-Asp-Gly-Ser-Thr-Asp - $\frac{m}{e}$ 929

Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr - $\frac{m}{e}$ 1151, molecular ion peak.

Above 900 the peaks were very small. Peaks were observed at $\frac{m}{e}$ 199, 328, 657 and 786 but not at 471 or 542. If the Asp next to Gly was in the αβ form and cleavage across both carbon-nitrogen bonds in the succinimide ring occurred then a peak would be expected at $\frac{m}{e}$ 440, and indeed, there was such a peak. The following peaks would then be expected:- $\frac{m}{e}$ 496, 611, 611, 740 and 883 but all of these were absent.

Peaks at $\frac{m}{e}$ 121 and 192 were observed and are indicative of C-terminal tyrosine, the former peak being produced by the tyrosine side chain. The peak at $\frac{m}{e}$ 199 is characteristic of N-terminal asparagine. Peptide impurities in the sample were easily observed, for instance peaks at $\frac{m}{e}$ 170, 142 and 100 signifying N-terminal leucine (or isoleucine).

The spectrum indicated that there was some sort of irregularity

in the peptide around the aspartyl residue next to glycine.

The structure of peptides containing arginine (or histidine or the sulphur containing amino acids) cannot be fully elucidated by mass spectrometry using direct N-acetylation and permethylation. Arginine containing peptides may be treated with acetyl-acetone[88] or cyclohexane-1,2-dione[89,90] before acetylation and permethylation. For untreated peptides the sequence ions from the N-terminus to the residue prior to arginine are observed. No sequence peaks at all are seen if the other 'difficult' amino acids are present. In this study arginine was the only 'difficult' amino acid expected and since it was always C-terminal, the peptide was not modified prior to derivatisation. The only peak absent from the spectrum should be the molecular ion peak.

The arginine containing peptides Ly1T12 and Ly2T12 were analyzed but in neither case was the resultant trace satisfactory. In the former case no sequence peaks were observed. In the latter case a peak was seen at $\frac{m}{e}$ 199 indicating N-terminal asparagine but 342 and 413 peaks representing Asn-Asp and Asn-Asp-Gly respectively were absent. Also absent were peaks corresponding to the imide form of aspartic acid.

However peaks were observed at $\frac{m}{e}$ 98, 126, 253, 409 and 524. The 126 peak and 98 peak refer to pyroglutamic acid derived from cyclization of the N-terminal glutamic acid (or glutamine) accompanied by chain cleavage and, in the case of $\frac{m}{e}$ 98, loss of carbon monoxide. The peak at $\frac{m}{e}$ 253 corresponds to a difference of 127 mass units over $\frac{m}{e}$ 126 and is indicative of leucine or isoleucine. Next is $\frac{m}{e}$ 409, 153 mass units greater than $\frac{m}{e}$ 253, which refers to asparagine and so gives the sequence Glx-Leu (or Ile)-Asn. $\frac{m}{e}$ 524 is 115 mass units greater than 409 and corresponds to serine. No other sequence peaks were observed at higher masses. The structure would appear to be Glx-Leu(or Ile)-Asn-Ser. $\frac{m}{e}$ 146, referring to C-terminal serine, was not observed. In the lysozyme structure there is the sequence Gln-Ile-Asn-Ser-Arg which is preceeded by

leucine. Since leucine is susceptible to C-terminal cleavage by chymotryptically active enzymes, the sequence could easily have been cleaved during the enzymic digestion. From the amino acid analysis of this peptide fraction these residues are in a minority and so this penta-peptide must be a contaminant of the tetrapeptide Asn-Asp-Gly-Arg. The reason why the impurity was sequenced whilst the major component was not, is unknown, but this situation can occur when the major component is in some way 'difficult'.[91] Variation of probe temperature can be used to analyze peptide mixtures by mass spectrometry.

The amino acid sequence immediately prior to LyT12 is Arg-Trp-Trp-Ae-Cys and all these peptide bonds are potential points of cleavage for the trypsin sample used. However, probably because of steric reasons, the enzyme is unlikely to cleave them all. Cleavage will occur after arginine and, in the case of Ly2, there seems to be cleavage after β-aminoethyl-cysteine. The amino acid analysis of LylT12 indicated a much higher concentration of β-aminoethyl-cysteine than was the case for Ly2T12. This could be explained by the presence of the peptide LyC_3T7 (i.e. Val-Ae-Cys) and the peptide LyT24 (i.e. Gly-Ae-Cys), but another explanation is that cleavage occurred not after aminoethyl-cysteine but after the second tryptophan residue and the penta-peptide Ae-Cys-Asn-Asp-Gly-Arg resulted. The presence of β-aminoethyl-cysteine would prevent any sequence peaks being observed in mass spectrum. The peptide should be treated with Raney Nickel[92] to desulphurize the residue before derivatisation. In the LAP digestion of LylT12, the presence of aminoethyl-cysteine was noted but it would also be present by cleavage of the Val-Ae-Cys and Gly-Ae-Cys bonds. A dansyl-Edman N-terminal determination was performed but the results were not clear. However on thin layer electrophoresis at pH 6.5

this peptide was shown to migrate to the anode, whilst a sample of Ly2T12 under the same conditions remained near to its starting position. The peptide under discussion would appear to be basic and so is likely to be Ae-Cys-Asn-Asp-Gly-Arg.

Samples of two more batches of the octapeptide given earlier, $Ly2C_1T10$ and $Ly3C_1T10$ were analyzed. For the first of these peptides, a peak at $\frac{m}{e}$ 199 indicating N-terminal asparagine and peaks at $\frac{m}{e}$ 192 and 121 indicating C-terminal tyrosine were observed. The sequence ion at $\frac{m}{e}$ 328 corresponding to Asn-Thr was seen but no other sequence peaks were observed. All the peaks at $\frac{m}{e}$ >600 were very small. The results were similar for the other peptide the only peaks of interest being at $\frac{m}{e}$ 121, 192, 199 and 328. In both cases no peak at $\frac{m}{e}$ 440 was observed corresponding to cleavage of the αβ-Asp imide ring.

The reason for the lack of appropriate peaks could be only partial derivatisation of sample or perhaps an intrinsic difficulty in sequencing rearranged, or partially rearranged, Asp-Gly sequencies. However the final two peptides present were in the natural state before methylation and analysis. Since the sequence of peptide $Ly2C_3T10$ could be correctly determined, the modification reagents would appear to have been in good condition.

Electrophoresis

The components of peptide $Ly2C_1T10$ were separated electrophoretically using both paper and thin layers of cellulose. The pH used was 3.5 and it was hoped to separate the α-aspartyl and β-aspartyl peptides and investigate any rearrangement in the N-terminal asparagine.

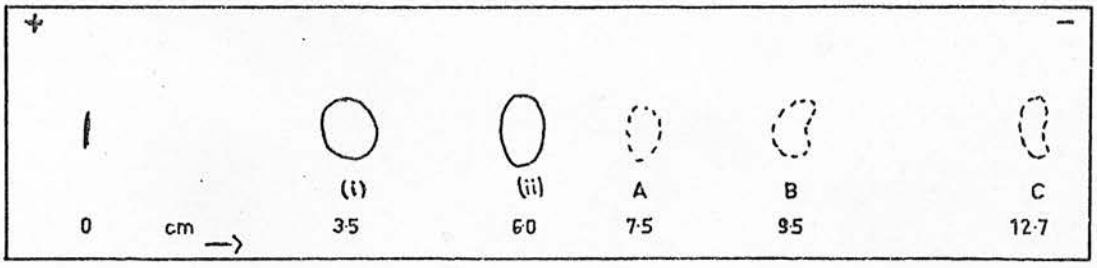
Paper electrophoresis run 1 was purely analytical and the results are shown in table 10.10. The three pale purple spots, A, B and C, are small

Table 10.10

Peptide $Ly2C_1T10$:- Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr

Electrophoresis

(A) Analytical Paper Electrophoresis



(B) Preparative Paper Electrophoresis

	Corrected Peak Areas				
Spot	Asp	Asn	Gly	Thr	Ser
Fast (ii)	-	0.37	0.35	0.01	-
Slow (i)	0.01	-	-	-	-

(C) Preparative Thin Layer Electrophoresis

	Corrected Peak Areas				
Spot	Asp	Asn	Gly	Thr	Ser
Fast (ii)	0.27	3.51	3.40	0.28	0.18
Slow (i)	2.04	-	-	-	-

amounts of peptide impurity present in the peptide. However spots (i) and (ii) were both strong, purple in colour and of approximately equal intensity. There are four possible explanations:-

(a) The aspartic acid next to glycine is approximately 50% rearranged so that equal amounts of α- and β- aspartyl peptides are being observed. However this seems unlikely since the separation is large and the two peptides differ only slightly in their degrees of acidity.

(b) The aspartic acid has rearranged to the αβ-aspartyl group. This would allow the rearranged peptide to travel faster than the normal peptide and could explain the positions of the two spots.

(c) If the N-terminal asparagine was partially rearranged to form a β-aspartyl unit or simply deamidated to an α-aspartyl group the two spots would be expected. The deamidated peptide would be the slower of the two.

(d) If the alleged aspartyl group next to glycine is, in fact, an asparaginyl group, then rearrangement of this unit to an α- or β-aspartyl unit, with loss of ammonia,[93] would lead to the formation of two spots. Canfield[23] postulated aspartic acid for this position but Jollès et al[94,95] suggested that this peptide might contain asparagine next to the glycine and be N-terminal in aspartic acid. However work done in this laboratory using LAP and Edman degradation, especially the latter, substantiated Canfield's postulated sequence. Despite the fact that spots corresponding to DNS-Asp-and DNS-Asn can sometimes be confused on TLC plates, especially if streaking has occurred, it would appear that the structure of this peptide is as originally stated. This explanation for the formation of the two

electrophoresis spots then, is therefore unlikely.

The LAP digest results from the preparative samples are given in table 10.10. In the paper electrophoresis the slow component contains no asparagine and only a trace of aspartic acid, suggesting that the N-terminal asparagine has rearranged to some extent to the β-aspartyl form. The fast component showed the presence of asparagine, but aspartic acid was absent. The thin layer electrophoresis results were similar, except that the fast component contained a little aspartic acid. However, because of the low level of peptide used in these experiments, the results should be viewed qualitatively rather than quantitatively. Despite this, they suggest that the N-terminal asparagine in this peptide is sufficiently labile to undergo a partial rearrangement to the β-aspartyl form. From these, and the LAP digestion results, it can be seen that this N-terminal asparagine rearrangement is not complete.

The α- and β-aspartyl-glycyl peptides were not separated.

Induced Asparaginyl Rearrangement in the Synthetic Peptide, S1

The results are shown in tables 11.2-10 and figures 11.1-6. Using pyridine acetate buffer (0.1M in base) to induce the rearrangement (table 11.2) it was seen that rearrangement was a minimum at around pH5 and increased at both higher and lower pHs. The increase in reaction at low pH was only small but it increased rapidly at higher pH. A similar result was observed with the buffer that was 5M in base (table 11.4). In table 11.3 the pH of the pyridine acetate buffer system was kept at a constant value of 5.0 and the concentration of base varied. Up to a concentration of 2M the degree of rearrangement varied little but at the high concentrate of 5M an increase in the rearrangement degree

was noted. However, it was still almost a quarter of that for the 0.1M buffer at pH 8.2 indicating that pH is much more important than concentration in inducing the rearrangement. In all three cases the level of aspartic acid observed was very small and was not directly proportional to either concentration or pH. Since the level of aspartic acid remained low throughout, a simple loss of ammonia from the amide is not taking place; this would lead to α-aspartic acid which would be cleaved by LAP.

In table 11.5 an aqueous solution 1M in pyridine was used at a pH of 8.6 and the temperature of incubation was varied. As might be expected the degree of rearrangement increased with increase in temperature, but even at 4°C it was still quite substantial. The asparagine concentration for the 75°C sample is probably higher than it should be since the solvent completely evaporated during the second overnight period. It would appear that lowering the temperature does not substantially affect the position of equilibrium of the rearrangement but it, no doubt, affects the rate.

In table 11.6 the organic buffers were used as 1M aqueous solutions and were not buffered to a constant pH. As expected pyridine gave a mixture with a low asparagine concentration and an even lower aspartic acid concentration. Triethylamine behaved similarly, as did 2,6-lutidine, but here the asparagine concentration was quite high. The aspartic acid concentration was also higher than normal and it would seem that 2,6-lutidine is less efficient at inducing the rearrangment than the other bases tested. Ammonia gave very low value of both asparagine and aspartic acid. Trimethylamine and N-methylmorpholine both gave low values of asparagine but much higher values of aspartic

acid. It would appear that as well as the rearrangement process, loss of amide ammonia from the asparagine residue is taking place to give α-aspartic acid which is susceptible to the action of LAP. Alternatively these basic solutions could be acting to promote the back reaction in the α-Asp⇌β-Asp rearrangement. The former explana- is probably more likely. The difference in behaviour cannot be explained simply in terms of differences in pH, pka or dielectric constant of the bases, but no doubt all these three factors are involved.

In table 11.8 the rearrangement was monitored against time. It was observed that the concentration of asparagine fell exponentially and seemed to reach an equilibrium level. The concentration of aspartic acid remained low throughout and did not vary consistantly with time. Consider the reaction:-

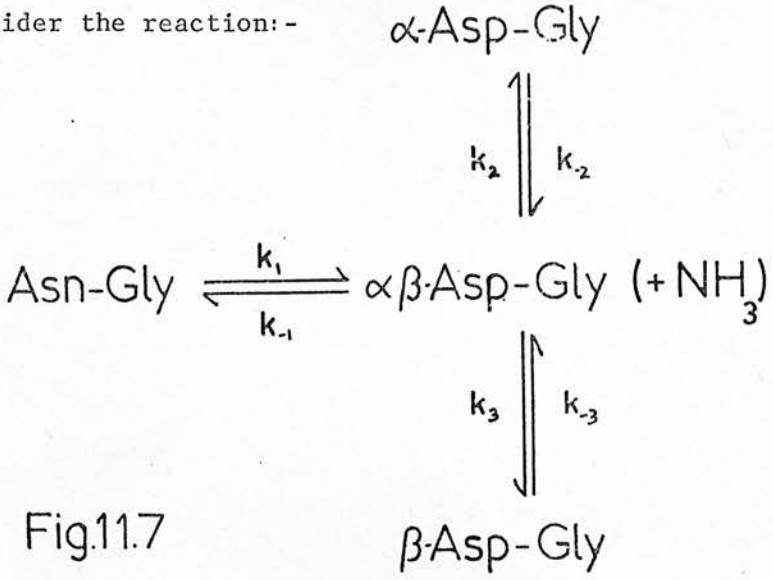


Fig.11.7

If initially $k_1 >> k_{-1}$ then the early stages of the reaction may be a simple first or second order plot. For a first order plot:-

Rate of decrease in $[Asn] = \frac{-d}{dt}[Asn]$

If at t = o $[Asn] = a$, and at time, t, $[Asn] = (a-x)$, then

Rate $= \frac{dx}{dt} = k(a-x)$ where k = rate constant

$$\frac{dx}{(a-x)} = kdt$$

integrating

$$-\ln(a-x) = kt + \text{Constant}$$

Now, when $t = o$, $x = o$

$$\text{Constant} = -\ln a$$

$$-\ln(a-x) = kt - \ln a$$

Thus a graph of $\ln(a-x)$ against t will give a straight line graph in the case of a first order reaction and from the slope of the line the rate constant, k, can be found.

Initially this graph was found to be straight line and the rate constant was calculated to be $1.1 \pm 0.02 \times 10^{-4} \text{sec}^{-1}$.

$$\underline{\underline{k = 1.1 \times 10^{-4} \pm 0.02 \times 10^{-4} \text{ sec}^{-1}}}$$

For a second order plot

$$\text{rate of decrease of } [\text{Asn}] = \frac{-d}{dt}[\text{Asn}] = k''[\text{Asn}]^2 = k''(a-x)^2$$

$$\frac{dx}{dt} = k''(a-x)^2$$

$$\frac{dx}{(a-x)^2} = k''dt$$

$$\frac{1}{(a-x)} = k''t + P$$

now when $t = o$, $x = o$, $P = \frac{1}{a}$

$$k''t = \frac{x}{a(a-x)}$$

There is not now an exponential variation of x with t. A second order plot was drawn (fig. 11.5c) from the data given in table 11.10. Since the base or buffer is in large excess its concentration has not been considered in these kinetic equations.

Returning to fig. 11.7, since LAP will not cleave αβ-aspartyl peptides or β-aspartyl peptides then the aspartic acid observed must

have come from the α-aspartyl-glycine. However the level was always small and showed no obvious trend so it can be assumed that k_2 and k_{-2} are small and approximately equal. In the later stages of the kinetic run it can be seen (Fig. 11.5a) that reaction 1 is reversible since an equilibrium level of asparagine has been reached. The situation is probably complicated by the fact that the liberated ammonia may not all be staying in solution since the reaction mixture is at pH 8.6 and 50°C. The first order plot gave a straight line initially (fig. 11.5b). A second order plot also gave a straight line in the initial stages of the reaction but only after a lag time of about 40 minutes. Agreement with theory was certainly no better than with the first order plot. It would appear then that reaction 1 is initially first order and reaction 2 occurs to only a small degree. From the literature it would appear that the αβ-aspartyl structure is not very stable in an alkaline medium[5] and so β-aspartyl-glycine would appear to be the major product.

Consider the following simplified reaction scheme for the initial reaction:-

$$\text{Asn} - \xrightarrow{k_1} \alpha\beta\text{-Asp-} \xrightarrow{k_3} \beta\text{-Asp-}$$

representing this as $A \xrightarrow{k_1} B \xrightarrow{k_3} C$

Rate of disappearance of A is

$$-\frac{d[A]}{dt} = k_1[A] \qquad \text{(i)}$$

Rate of appearance of B is

$$\frac{d[B]}{dt} = k_1[A] - k_3[B] \qquad \text{(ii)}$$

Rate of appearance of C is

$$\frac{d[C]}{dt} = k_3[B] \qquad \text{(iii)}$$

From (i)

$$\frac{d[A]}{[A]} = -kdt$$

and $[A] = ae^{-k_1 t}$ since $[A] = a$ when $t = o$

in (ii)

$$\frac{d}{dt}[B] = k_1 ae^{-k_1 t} - k_3[B]$$

$$[B] = a\frac{k_1}{k_3 - k_1}(e^{-k_1 t} - e^{-k_3 t})$$

Since $[A] + [B] + [C] = a$

then $[C] = a - [A] - [B]$

$$= a\ 1 - \frac{k_3 e^{-k_1 t} - k_1 e^{-k_3 t}}{k_3 - k_1}$$

The data available only measured $[A]$ and $[B]$ and $[C]$ were not monitored. These equations can only be used to find k_3 if a method is found for the quantitative detection of $[B]$ or $[C]$.

If the following reaction is considered:-

Asn- $\underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}}$ αβ-Asp- $\xrightarrow{k'_3}$ β-Asp-

representing this by $A \underset{k'_1}{\overset{k'_1}{\rightleftharpoons}} B \xrightarrow{k'_3} C$

rate of disappearance of A is

$$\frac{-d}{dt}[A] = k'_1[A] - k'_{-1}[B]$$

rate of appearance of B is

$$\frac{d}{dt}[B] = k'_1[A] - k'_{-1}[B] - k'_3[B]$$

rate of formation of C is

$$\frac{d}{dt}[C] = k'_3[B]$$

If B - the imide - is in low concentration, then the steady-state approximation will hold and:-

$$\frac{d\,B}{dt} = k_1' A - k_{-1}' B - k_3' B = o$$

$$B = \frac{k_1' A}{k_{-1}' + k_3'}$$

and

$$\frac{d\,A}{dt} = \frac{d\,C}{dt} = \frac{k_3' k_1' A}{k_{-1}' + k_3'} \qquad (a)$$

if $k_3' >> k_{-1}'$ then we have $\frac{d\,A}{dt} = k_1' A$ ie, an apparent first order rate.

It has been assumed that ammonia has not been lost from the system and is not involved in the system kinetics.

From equation (a), the first order rate constant calculated previously, k is in fact equal to $\frac{k_3' k_1'}{k_{-1}' + k_3'}$. To find values for k_1', k_{-1}' and k_3' values for $[B]$ and $[C]$ must be found.

To solve the kinetic problems of this system, then, the concentrate of imide or the β-aspartyl peptide must be ascertained. Since the sum of the concentrations of the four peptides involved in this rearrangement is constant both imide and β-aspartyl peptide need not be found. Determination of the concentration of the imide using IR spectrometry by monitoring the change in intensity of the imide carboxyl groups is difficult because of the use of aqueous solutions. However the use of Raman spectroscopy is worth investigating since aqueous solutions pose no problems here. Titration of the free carboxyl groups in α-aspartyl and β-aspartyl residues by means of an autotitrator is possible and since the amount of the α-isomer can be observed from the LAP analysis, by difference, a measure of the β-aspartyl concentration can be obtained. However this method may well lack sensitivity and possess technical

problems. It may also be complicated by loss of ammonia.

From table 11.7 it is clear that acetic acid has much less effect on the rearrangement process than base. The 1M aqueous acetic acid at the low pH of 2.5 had hardly any effect on the asparagine concentration and even glacial acetic acid produced less than 50% rearrangement. Aspartic acid concentration was again low.

From table 11.9 it can be seen that only 1 peak was observed when S1 was chromatographed and 10 µl of sample gave an aspartic acid concentration of 9.25×10^{-2} µmoles after acid hydrolysis. The difference between this value and the combined concentration of aspartic acid and asparagine from the t=o LAP digest, gave a value for the degree of rearrangement caused by the basic conditions of the LAP digest. It was observed to be quite small.

A partial digest run was performed and peaks representing aspartic acid, asparagine, glycine and the internal standard valine were observed. Also, three broader peptide peaks were noted following valine and not fully resolved. The first of these three peaks had a high 440 nm absorption value and so is probably Asn-Gly-OEt. The other two peaks probably represent αβ Asp-Gly and β-Asp-Gly with possibly some α-Asp-Gly present as well.

Separation of the non-digested peptide after treatment with 1M pyridine using the conditions of Dorer et al[25] gave a chromatogram consisting of five very small peaks (excluding valine) and one very broad peak immediately prior to the elution of valine. The peaks could not be characterized.

Induced Rearrangement in the Natural Product Peptides

The results are given in tables 11.11 & 12 and fig. 11.6. It can

be seen that the action of 5M aqueous pyridine (pH 8.6) with the peptide $Ly2C_1T10$ caused a rapid decrease with time in the amount of aspartic acid liberated and this corresponds to a high rate of aspartyl rearrangement. Thereafter the concentration remained relatively static. This static level may be due to the equilibrium

$$\alpha\text{-Asp} \rightleftharpoons \alpha\beta\text{-Asp} \rightleftharpoons \beta\text{-Asp}$$

or it may be due to a background level of aspartic acid impurity in the sample. The reaction with 0.1M aqueous pyridine solution gave a much slower rate of rearrangement than was the case with the 5M base but it was still significant.

From the results with the synthetic peptide, S1, it was seen that pH is the most important factor in determining the rate of rearrangement. The natural product peptides were air dried by removing the buffer solvent with compressed air. The pH of the initial solvent was known but not the pH of the solution at various stages of concentration. Buffers of pH 2.5, 3.1, 3.7 and 5.0 were dried down and their pHs measured after evaporation of 1 ml fractions. In all cases the low pH buffers tended to increase in pH and the higher ones decrease in pH such that a final pH of between 2.6 and 4.2 was obtained. Thus air drying should not induce rearrangement by subjecting the peptide to very high or very low pH with these buffer systems.

The data obtained from these investigations with both synthetic and natural product peptides can be used to reappraise the isolation procedure for natural product peptides. Avoidance of high pH is most important. The column separation procedure employing pyridine buffers would seem to be satisfactory since the pH was never greater than 5.0 and only very basic peptides needed the 5M buffer.

In the tryptic digests the enzyme has its optimum activity in the pH range 7.8 - 8.2 and to keep rearrangement to a minimum the lower of these two values should be used. The disulphide bond cleavage and aminoethylation procedures used buffered solutions at pH 8.5. This may not be a serious as it suggests since LAP digestions were performed at pH 8.6 in N-methyl-morpholine acetate buffer and only slight rearrangement took place. Once isolated, the peptide should be kept in a dry condition until required for investigation.

Table 11.2

Induced Asparaginyl Rearrangement in S1 I — See fig.11.1

0.1M Pyridine Acetate buffers : 50°C : 42 hrs. incubation

Buffer pH	Total Amount of Amino Acid Present [Asn] $\times 10^{-2}$ µmoles	[Asp] $\times 10^{-2}$ µmoles
3.1	7.04	0.33
5.0	7.21	0.49
5.9	5.54	0.39
7.0	*	*
8.2	1.18	0.16

Table 11.3

Induced Asparaginyl Rearrangement in S1 II — See fig.11.2

Pyridine Acetate buffers pH 5.0 : 50°C : 42 hrs. incubation

Base Concentration of Buffer	Total Amount of Amino Acid Present [Asn] $\times 10^{-2}$ µmoles	[Asp] $\times 10^{-2}$ µmole
0.1 M	7.21	0.49
0.5 M	7.52	0.50
1.0 M	6.25	0.41
2.0 M	7.00	0.39
5.0 M	4.05	0.01

Table 11.4

Induced Asparaginyl Rearrangement in S1 III — See fig. 11.3

Pyridine Acetate buffers, 5M in base : 50°C : 42 hrs incubation

	Total Amount of Amino Acid Present	
Buffer pH	[Asn] x 10^{-2} μmoles	[Asp] x 10^{-2} μmoles
4.7	2.63	0.14
5.0	4.05	0.01
5.9	3.91	0.35
7.0	1.09	0.14
8.6	0.30	0.08

Table 11.5

Induced Asparaginyl Rearrangement in S1 IV — See fig. 11.4

1.0 M pyridine, pH 8.6 : 42 hrs incubation

	Total Amount of Amino Acid Present	
Incubation Temperature	[Asn] x 10^{-2} μmoles	[Asp] x 10^{-2} μmoles
4°C	2.41	0
37°C	1.86	0
50°C	0.99	0
75°C	0.99*	0.31

*See text

Table 11.6

Induced Asparaginyl Rearrangement in S1 V

1M aqueous organic bases : 50°C : 42 hrs incubation

Base	pH	Total Amount of Amino Acid Present [Asn]x10^{-2}µmole	[Asp]x10^{-2}µmole
Pyridine	8.6	1.01	0.00
2,6-Lutidine	9.9	3.24	0.83
trimethylamine	11.7	0.47	1.59
triethylamine	12.2	0.82	0.15
Ammonia	11.4	0.14	0.16
N-methylmorpholine	10.3	0.02	1.19

Table 11.7

Induced Asparaginyl Rearrangement in S1 VI

Acetic Acid : 50°C : 42 hrs incubation

Acid Concentration	pH	Total Amount of Amino Acid Present [Asn] x 10^{-2}µmoles	[Asp]x10^{-2}µmoles
1M aqueous solution	2.5	7.65	0.31
Glacial (17.5M)	→ 0	4.44	0.36

Table 11.8

Induced Asparaginyl Rearrangement in S1 VII(a) See Figs.11.5a&b

1M Aqueous Pyridine pH 8.6 = 50°C

	Total Amount of Amino Acid Present		
Time of Incubation (h)	$[Asp] \times 10^{-2}$ μmole	$[Asn] \times 10^{-2}$ μmole	$Ln[Asn]$
0	0.43	7.48	-2.59
0.67	0.45	7.16	-2.64
1	0.25	5.46	-2.91
2	0.26	3.40	-3.38
4	0.32	1.50	-4.20
6	0.29	1.02	-4.59
8	0.14	0.02	-8.67
11	0.23	0.32	-5.75
24	0.14	0.61	-5.10
42	0.00	1.01	-4.60
48	0.29	0.96	-4.65

Table 11.9

Induced Asparaginyl Rearrangement in S1 VIII

	Total Amount of Amino Acid Present		
Run	$[Asp] \times 10^{-2}$ μmoles	$[Asn] \times 10^{-2}$ μmoles	Peptide
Peptide alone, no digestion	-	-	1 peak
No base, LAP digestion	0.43	7.48	-
No base, Acid hydrolysis	9.25		-

Table 10.10

Induced Asparaginyl Rearrangement in S1 VII(b) See fig.11.5c

Second order plot for kinetic run (table 11.8)

Total amount of Asn present x 10^{-2} μmole = (a-x)

At time t = o, x = o

Time of incubation, t(h)	$\frac{x}{a(a-x)}$
0	0
0.67	0.006
1	0.050
2	0.160
4	0.533
6	0.847
8	49.73
11	3.0
24	1.50
42	0.86
48	0.91

Table 11.11

Induced Aspartyl Rearrangement in $Ly4C_1T10$ I See fig.11.6

5M Aqueous Pyridine, pH 8.6 : 22°C

Incubation Period (mins)	[Asp] % original
0	100%
1	17½%
2	*
4	*
8	17%
16	15½%
32	14½%
64	19%

initial rate of rearrangement = 27% per sec $\pm$ 3% per sec.

Table 11.12

Induced Aspartyl Rearrangement in $Ly4C_1T10$ II See fig.11.6

0.1M Aqueous Pyridine, pH 8.2 : 22°C

Incubation Period (mins)	[Asp] % original
0	100%
1	100%
2	99%
4	88%
6	*
8	*
24	76%

initial rate of rearrangement = 2.2% per min. $\pm$ 0.25% per min.

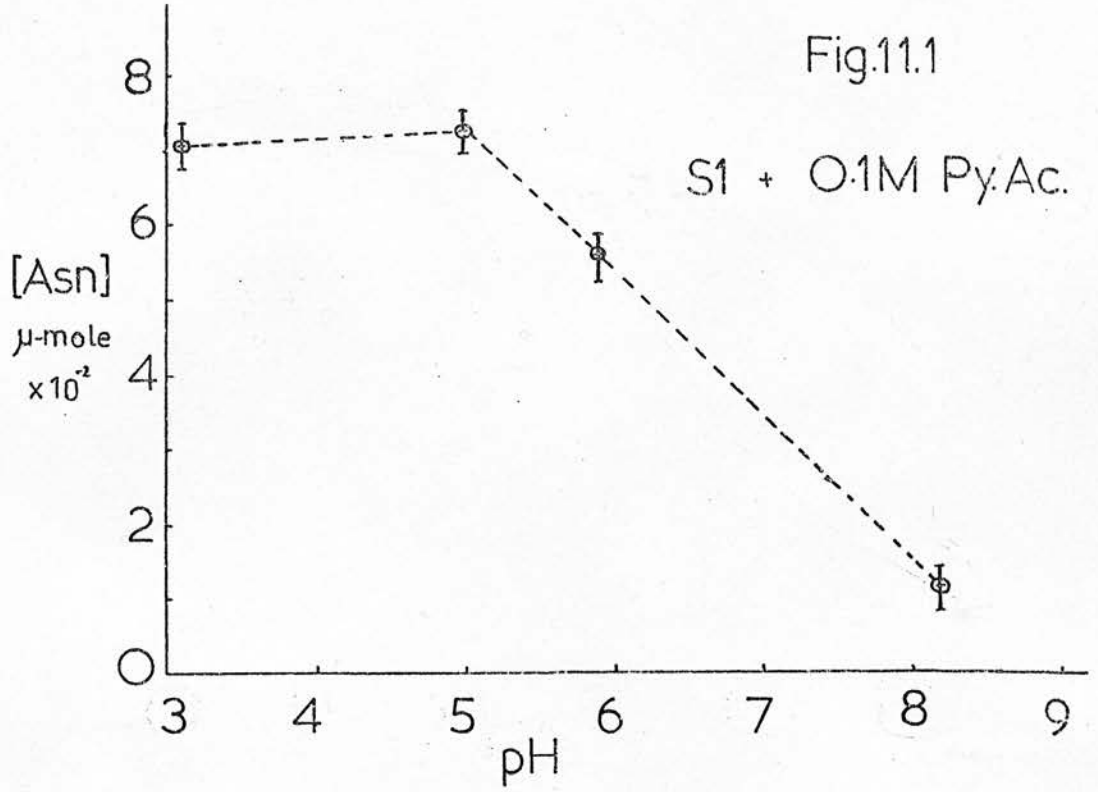


Fig.11.1
S1 + O·1M Py.Ac.
[Asn]
µ-mole
x10⁻²
8
6
4
2
0
3
4
5
6
7
8
9
pH

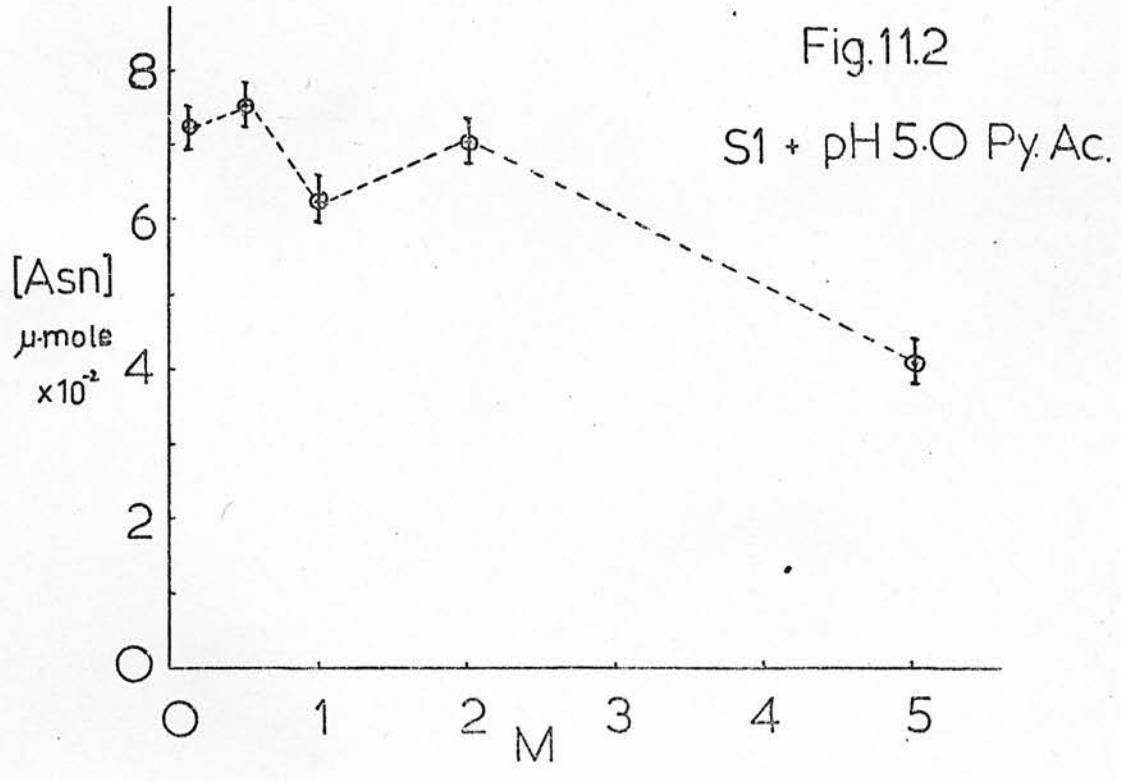


Fig.11.2
S1 + pH 5·0 Py. Ac.
[Asn]
µ-mole
x10⁻²
8
6
4
2
0
0
1
2
3
4
5
M

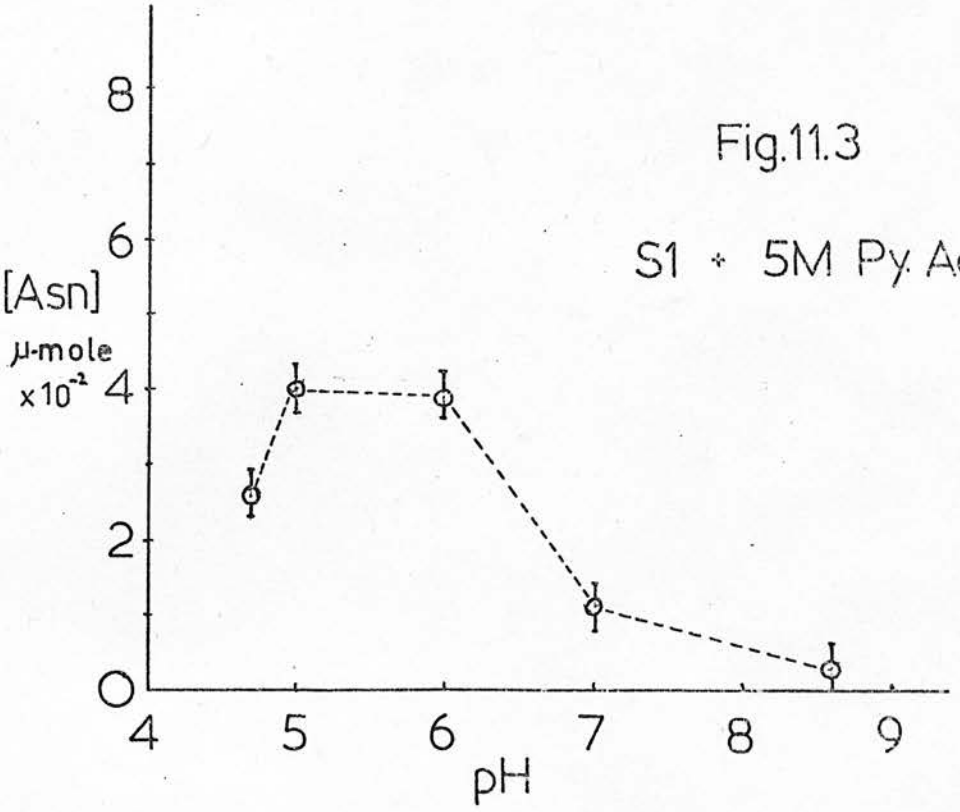


Fig.11.3

S1 + 5M Py. Ac.

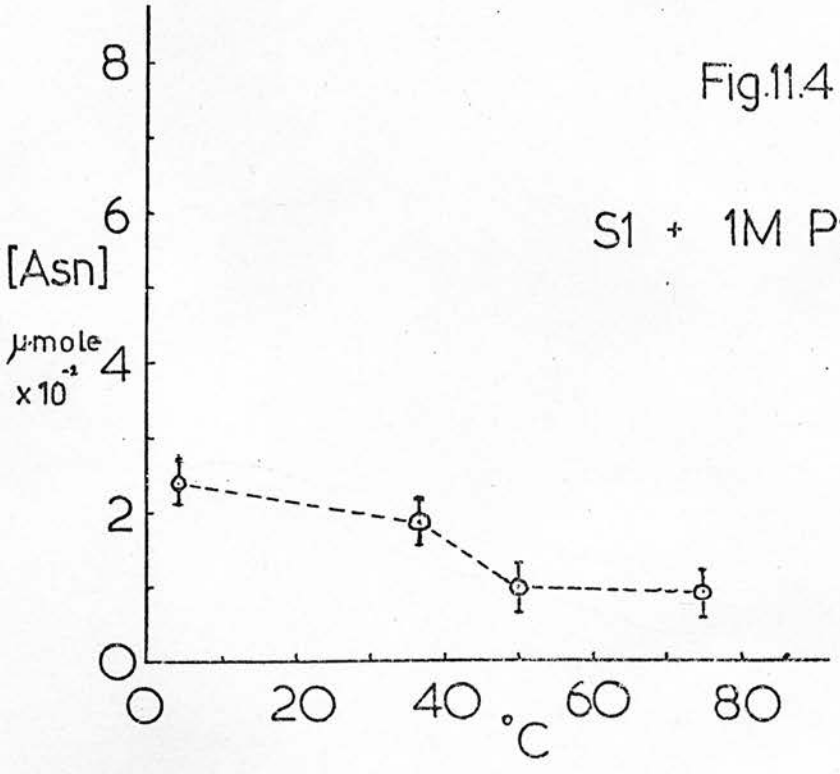


Fig.11.4

S1 + 1M Py.

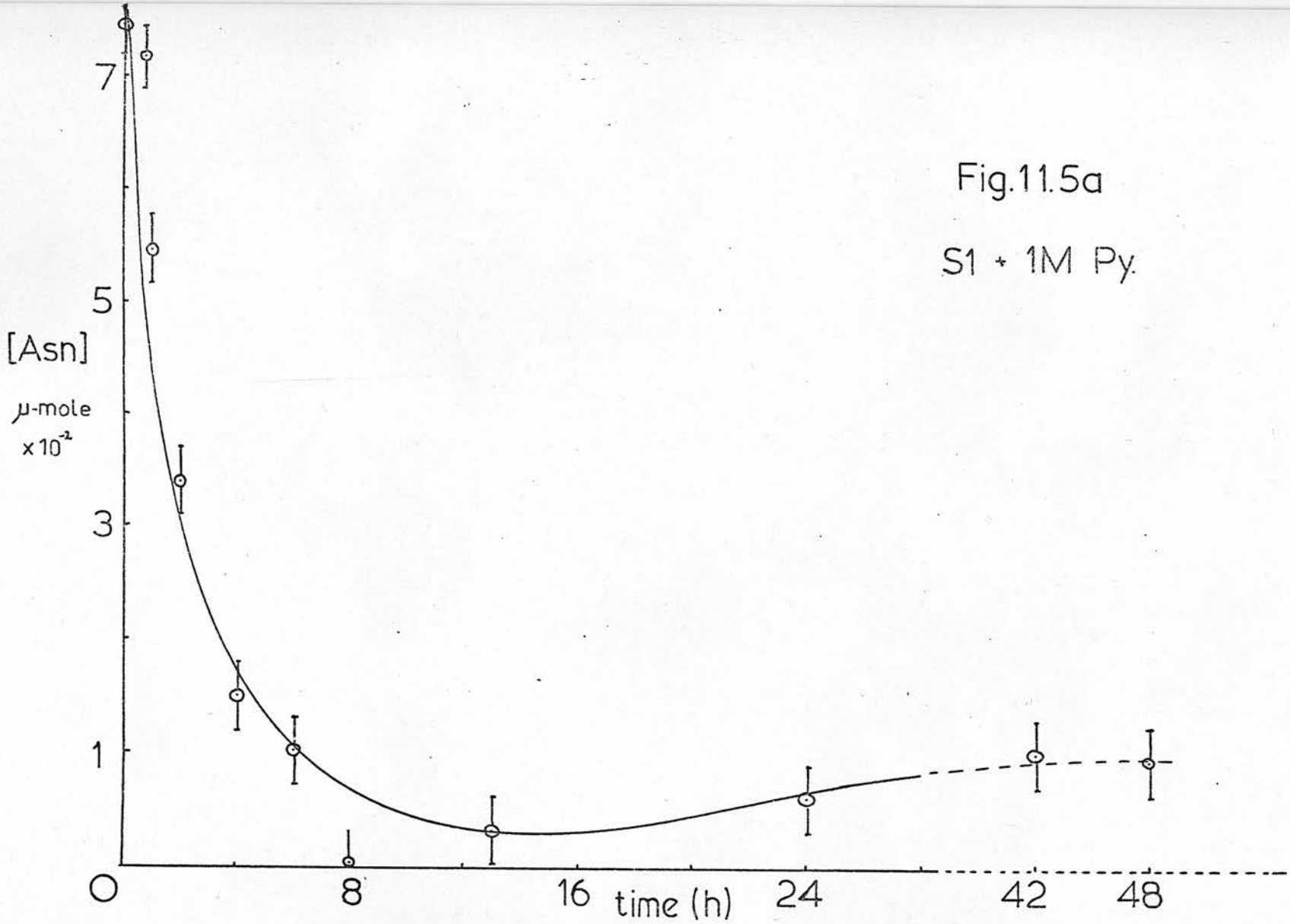


Fig.11.5a

S1 + 1M Py.

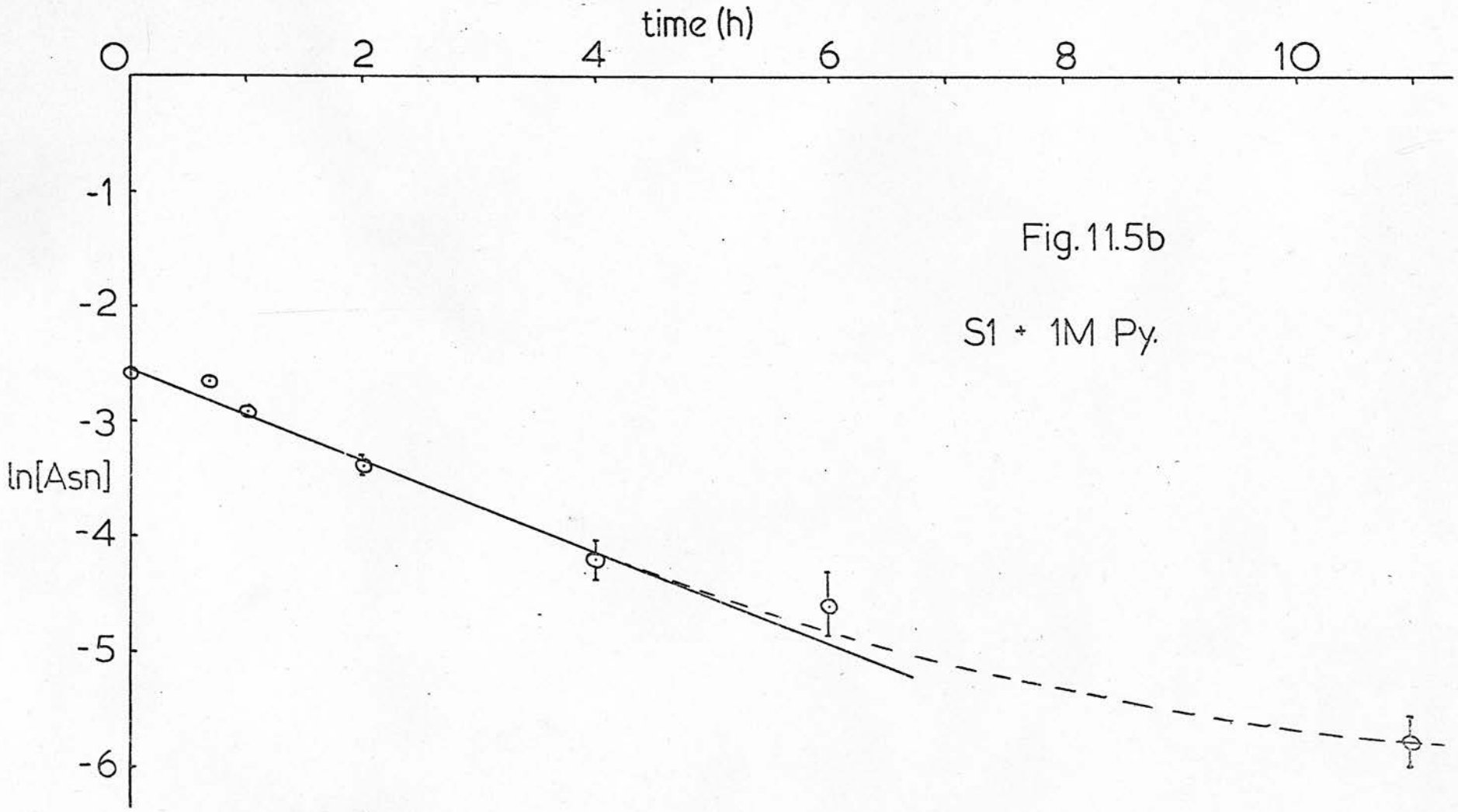


Fig. 11.5b

S1 + 1M Py.

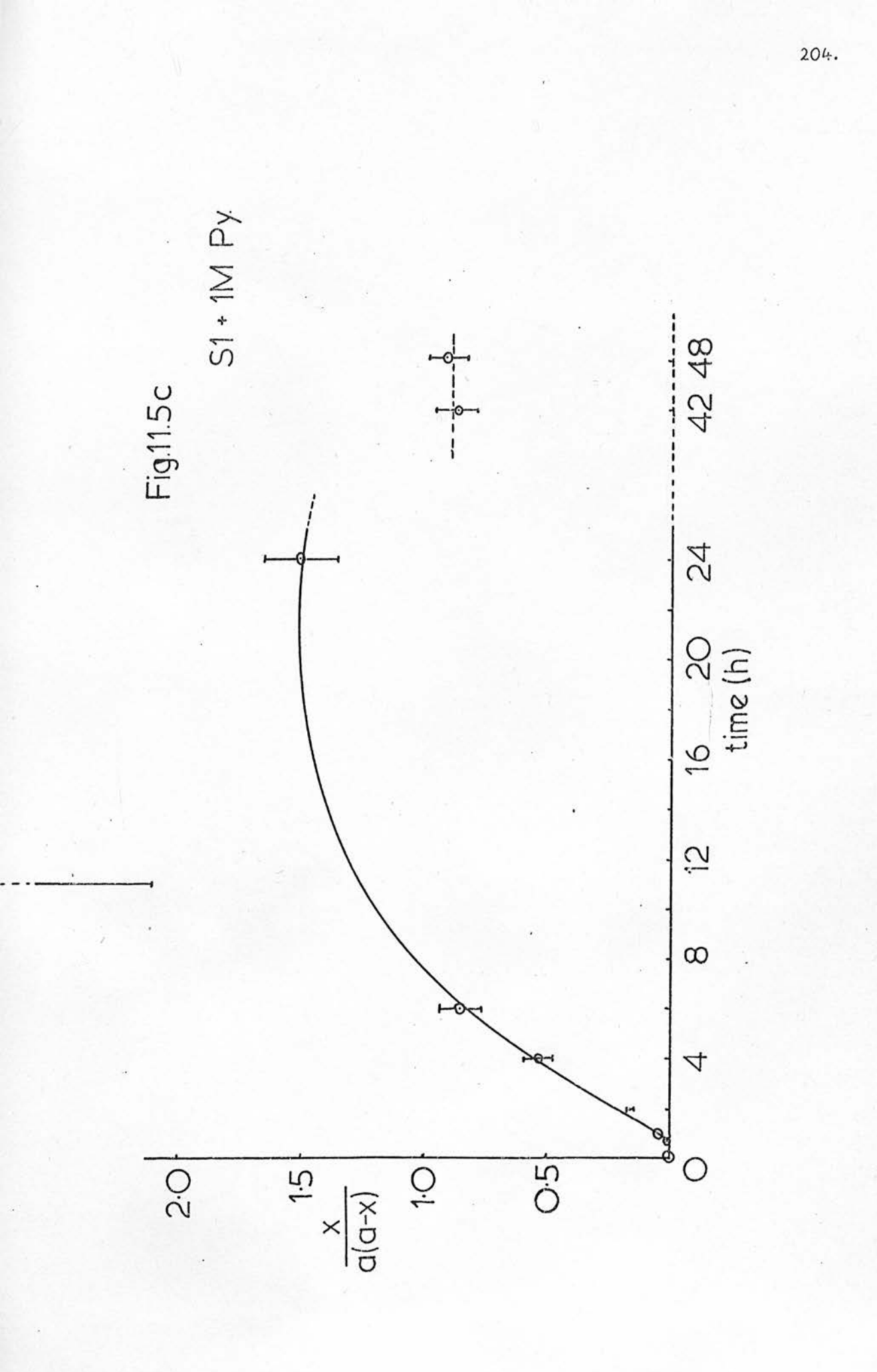
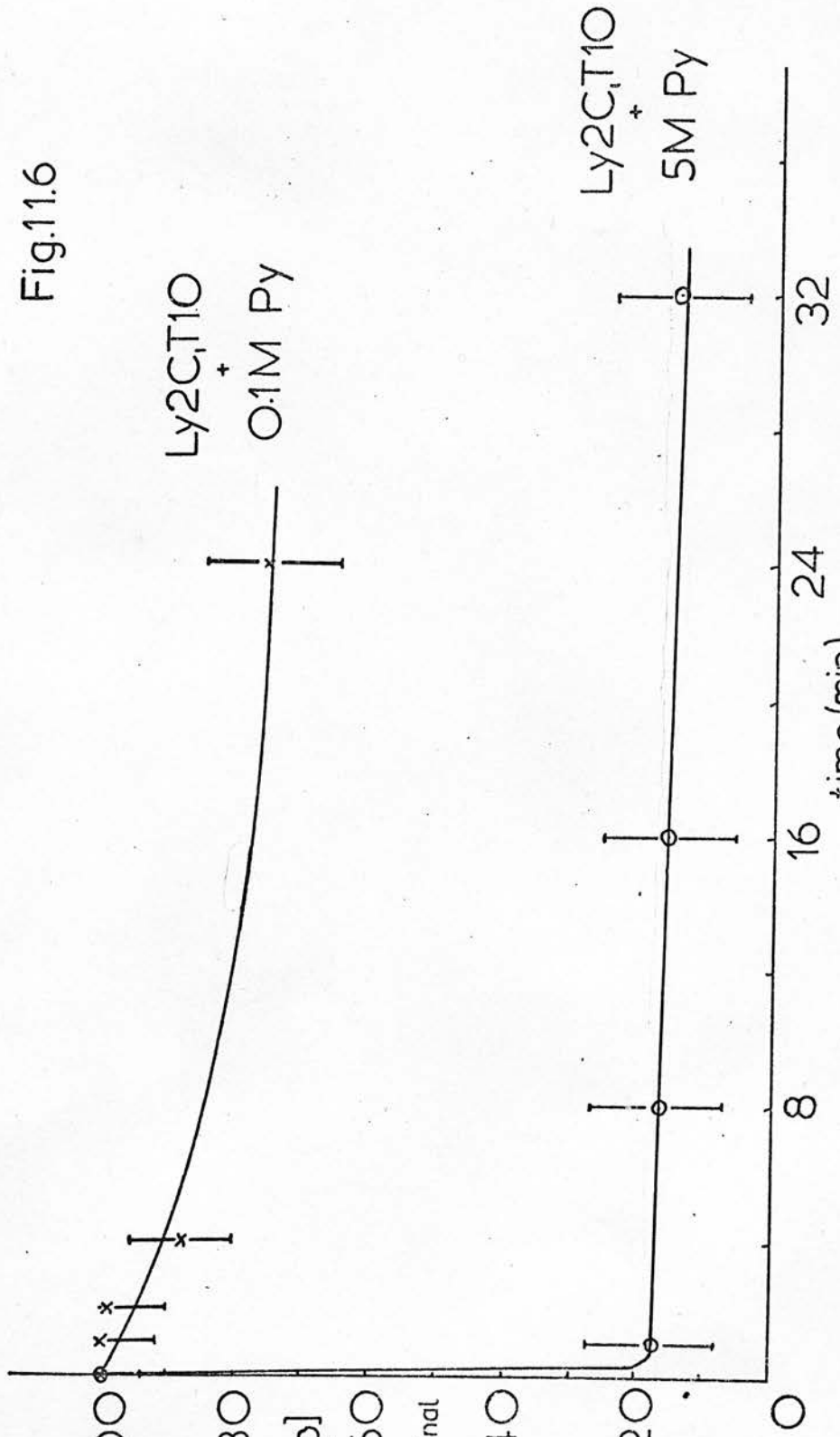


Fig.11.5c
S1 + 1M Py
2·0
1·5
1·0
0·5
0
$\frac{x}{a(a-x)}$
4
8
12
16
20
24
42
48
time (h)

Fig.11.6


Ly2C,T10
+
0·1M Py
Ly2C,T10
+
5M Py
100
80
60
40
20
0
[Asp]
% original
8
16
24
32
time (min)

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